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ENDOCRINOLOGY

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QUANTITATIVE ANALYSIS OF STEROIDS ISOLATED FROM THE URINE OF NORMAL AND ACTH TREATED GUINEA PIGS[1]

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ABSTRACT

The response of the guinea pig adrenal to intraperitoneal injections of ACTH is reflected by a four to eightfold increase in urinary corticoid and a one to twofold increase in urinary 17-ketosteroids. All the identified corticoids and 17-ketosteroids are eliminated in sufficient amounts to make their detection, isolation and quantitation possible by means of paper chromatography and conventional colorimetric assays. The corticoids and the 17-ketosteroid 6β,11β-dihydroxy-Δ^4-androstene-3,17-dione are eliminated in the urine as free compounds, while the ring A saturated 17-ketosteroids are eliminated as their water-soluble conjugated forms. Cortisone has been isolated for the first time from guinea pig urine.

THE isolation and identification of corticoids from the urine of normal and ACTH treated guinea pigs has been reported (1, 2, 3), whereas the quantitative estimation of these substances has only been partly determined by Burstein *et al.* (2). Several investigators have studied the amount of 17-ketosteroids eliminated daily in the urine of normal and scorbutic guinea pigs and in normal animals treated with ACTH. By applying the color correction equation of Talbot *et al.* (4) to values obtained by the colorimetric assay of crude extracts of acid hydrolyzed guinea pig urine, Clayton and Prunty (5) found that ACTH administration in the normal animal brought about a 40–206% mean increase in the amount of 17-ketosteroids eliminated in the urine. Oertel and Hein (6) reported urinary 17-ketosteroid values of 230 μg/day in the normal guinea pig and

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673 μg/day in the scorbutic animal following purification of ethyl ether extracts of acid hydrolyzed guinea pig urine on alumina columns. The much higher excretion values of the order of 500 μg/day and 1200 μg/day in the normal and ACTH treated animal, respectively, were reported by Bazzi and Nicoloci (7) who carried out their analyses on crude extracts of acid hydrolyzed guinea pig urine. No identification of the steroids obtained in these investigations was reported. Recently a study was carried out wherein several 17-ketosteroids were isolated from the urine of ACTH treated guinea pigs and identified (3). As a continuation of this work and of Burstein and coworkers' work (2), we wish to report in the present investigation a more complete quantitative picture of the urinary corticoids and 17-ketosteroids eliminated in the urine of normal and ACTH-treated guinea pigs.

MATERIALS AND METHODS

Urine collection and ACTH injection

Adult male guinea pigs (600–700 gm.) obtained from the Rockland Farms, New City, New York, were placed in metabolism cages and the urine collected as previously described (1). Prior to extraction the collected urine was stored in the frozen state. In all cases when ACTH (ACTHAR Gel, Armour) was used, 8 I U. were administered intraperitoneally to each animal for every day of urine collection.

Urine extraction, hydrolysis of the urine and purification of extracts

Pooled hydrolyzed and unhydrolyzed urine samples were extracted with ethyl acetate and further fractionated by methods previously described (1, 3). In this manner fractions A_1 and B_1 were obtained which represent organic solvent soluble material from unhydrolyzed and acid-hydrolyzed urine, respectively, following purification of these extracts on a silica gel column (cf. 3).

Comparative studies of 17-ketosteroid extraction from hydrolyzed urine was done with ethyl ether and carbon tetrachloride in addition to ethyl acetate. Extractions with ethyl ether (EtoEt) were carried out on urine which had been hydrolyzed by boiling 20 minutes in the presence of 10% HCl (v/v). The hydrolysate was extracted three times with twice its volume of EtoEt. The ether extract was washed twice with 10% NaOH (80 ml. NaOH/500 ml. of ether extract) and then with water until neutral.

For carbon tetrachloride extractions the urine was first acidified with acid (15% HCl, v/v), a ¼ volume of carbon tetrachloride (CCl_4) was added and the mixture refluxed on a hot plate for 30 minutes. The extraction was repeated once more with fresh CCl_4 following decantation of the first portion of CCl_4. The CCl_4 was pooled, washed with 10% NaOH (75 ml. NaOH/100 ml. CCl_4) and finally with water until neutral. Subsequently, both the EtoEt and CCl_4 extracts were treated in the same manner as the ethyl acetate extracts prior to paper chromatography.

Paper chromatography of fractions A_1 and B_1

Fractions A_1 and B_1 were chromatographed on paper using conventional solvent systems (8, 9, 10) and in the same sequence outlined previously (3).

Colorimetric assay of steroids eluted from paper chromatograms

Prior to the elution of the steroid zones on the chromatograms, individual corticosteroid zones were detected with the blue tetrazolium reagent (BT) and by visual exami-

nation of the chromatograms under ultraviolet light (Mineralight, Ultra-Violet Products, Inc., South Pasadena, California). The 17-ketosteroid zones were detected by development of the chromatograms with the Zimmermann reagent (9). Eluted corticoids bearing the 17α,21-dihydroxy-20-ketone grouping were analyzed quantitatively by the Porter-Silber method (11). Those not possessing this structure but having the α,β-unsaturated 3-ketone conjugated grouping were analyzed quantitatively by the phosphomolybdic reaction of Burstein (12). The 17-ketosteroids were analyzed quantitatively by the Holtorff and Koch method (13).

RESULTS

Table 1 is a summary of the results obtained when three different solvents were used for the extraction of the acid hydrolyzed urine. In order to eliminate variances in the amount of 17-ketosteroid excreted by different groups of animals, a pooled 140-day urine sample was obtained from 20 normal adult male guinea pigs and divided into four equal 35-day urine aliquots. Each aliquot was hydrolyzed with HCl and extracted with EtoEt, CCl_4 or ethyl acetate, respectively (cf. methods). Following the control

TABLE 1. QUANTITATIVE ANALYSIS OF 17-KETOSTEROIDS ELUTED FROM PAPER CHROMATOGRAMS OF ETHYL ETHER, CARBON TETRACHLORIDE AND ETHYL ACETATE EXTRACTS OF ACID HYDROLYZED URINE

Solvent used for extraction	Aliquot days	ACTH injected per animal I.U./day	μg./day 17-KS	% increase over control
Ethyl Ether	35	—	101	—
Ethyl Ether	35	8	504	400
Carbon Tetrachloride	35	—	30	—
Carbon Tetrachloride	35	8	152	407
Ethyl Acetate (a)	35	—	95	—
Ethyl Acetate (b)	35	—	121	—
(a)	35	8	412	334

collection period the same animals were injected with 8 I.U. of ACTH per day for a period of 7 days, the urine collected, pooled, aliquoted, hydrolyzed and extracted as for the control urine. The individual extracts were next subjected to silica gel chromatography to yield purified fractions B_1. Following paper chromatography of fractions B_1 all eluted 17-ketosteroid zones of the paper chromatograms were pooled and quantitatively analyzed. The results obtained (Table 1) indicate that both EtoEt and ethyl acetate extract approximately the same amount of 17-ketosteroids from the acid hydrolyzed urine. Carbon tetrachloride was not as efficient a solvent for extracting the urinary 17-ketosteroids. As a result of these findings and since ethyl acetate is the solvent of choice for extracting corticoids quantitatively (1, 14), ethyl acetate was used in all subsequent experiments.

Urinary corticoids in control and ACTH treated guinea pigs

The findings of Burstein and Dorfman (2) and Péron and Dorfman (3, 15) are summarized diagrammatically in Figure 1 which shows the chroma-

tographic pattern of steroids which are extracted with ethyl acetate from non-hydrolyzed guinea pig urine and which are present in fraction A_1.

The usefulness of the chloroform-formamide (C-F) system is well-known, especially when used for the separation of mixtures of highly polar steroids. However, it is equally notorious in that it fails to give clean-cut separations and as a result the individual steroid zones are contaminated with the other zones found on the chromatogram. The failings of the above system are not experienced when the less polar toluene-propylene glycol (T-P-G) system (8) or the ligroin-propylene glycol (L-P-G) system of Savard (9) are used. For this reason and also to eliminate pigments which accompany some of the zones separated in the C-F system and interfere with the colorimetric assays, all steroid zones after elution were acetylated and rerun in the T-P-G system for varying lengths of time (cf. Table 2). In addition acetylation and rechromatography of C-F zone II in the T-P-G system is necessary in order to separate 2α-hydroxycortisol diacetate (II-Iac) from possible contaminating amounts of an unknown acetate (II-IIac) (cf. 16). Table 2 shows the amounts of corticoids[2] ob-

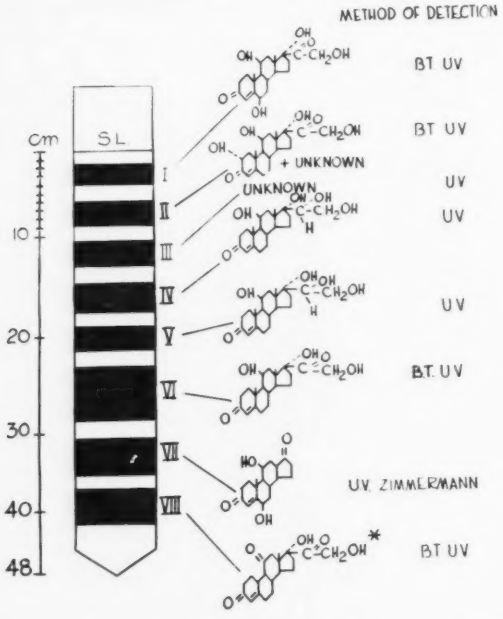

FIG. 1. Diagrammatical representation of a paper chromatogram of steroids extracted with ethyl acetate from non-hydrolyzed guinea pig urine after running in the chloroform-formamide system for 14 hours (C-F-14). S. L. = Starting line.

* Has not been reported previously.

[2] Zone VII although a 17-ketosteroid is included in Table 2 because it was isolated from fractions A_1 on the chloroform-formamide chromatograms along with the corticoids.

TABLE 2. QUANTITATIVE ANALYSIS OF CORTICOIDS ELUTED FROM CHLOROFORM-FORMAMIDE PAPER CHROMATOGRAMS AND RERUN AS THE ACETATES IN THE TOLUENE/PROPYLENE-GLYCOL SYSTEM

Zones from initial C-F chromatograms of fractions A_1	Running time of acetates run in the T-P-C-system hours	Name of steroid	μg./day control urine	μg./day ACTH urine	Response to ACTH % increase over control
I	Iac—16	6β-hydroxy cortisol diacetate	11.9	57.5	383
II	II-Iac—16	2α-hydroxy cortisol diacetate	23.3	47.5	104
	II-IIac*—16	unknown acetate	—	—	—
III	IIIac—8	unknown acetate	4.4	4.9	0
IV	IVac—8	20α-hydroxy cortisol diacetate	2.7	—	—
V	Vac—8	20β-hydroxy cortisol diacetate	1.4	13.8	885
VI	VIac—16	cortisol acetate	18.3	92.5	405
VII	VIIac†—16	6β,11β-dihydroxy Δ^4-androsten-3,17-dione-6-acetate	7.3	5.3	0
VIII‡	VIII—40	cortisone	2.7	12.5	363
Total excluding VIIac			64.7	228.7	

* Zone II-IIac as reported previously (16) was not found.

† Quantitative analysis of zone VIIac was carried out by the Holtorff-Koch procedure (13).

‡ Zone VIII was not acetylated and excretion values are reported on the free compound. Zone VIII was eluted from the initial C-F chromatogram and rerun in T-P-G for 49 hours prior to the assay.

tained in a 200 day equivalent sample of control urine and in a 216 day equivalent sample of urine of guinea pigs treated with ACTH. The response to ACTH injection is expressed in the percentage increase of corticoids excreted in the ACTH urine over the control values. It can be seen that ACTH causes an increased elimination of corticoids 6β-hydroxycortisol diacetate (Iac), 2α-hydroxycortisol diacetate (II-Iac), 20β-hydroxycortisol diacetate (Vac), cortisol acetate (VIac) and cortisone (VIII) in the order of 383, 104, 885, 405 and 363%, respectively. However, no increased excretion of IIIac (unknown acetate) and 6β,11β-dihydroxy-Δ^4-androsten-3,17-dione-6-acetate (VIIac) was found after ACTH injection.

Urinary 17-ketosteroids in control and ACTH treated guinea pigs

Figure 2 shows in a diagrammatical form the chromatographic pattern of the 17-ketosteroids which are extractable from acid hydrolyzed guinea pig urine and present in fraction B_1 (cf. 3). Table 3 shows the effect of ACTH injections on the urinary 17-ketosteroid output. Although both

11β-hydroxyandrosterone and $\Delta^{9,11}$-dehydroandrosterone were not detected in this investigation, they have been found in a previous study in very small amounts (3). Since $\Delta^{9,11}$-dehydroandrosterone is known to be a dehydration product derived from 11β-hydroxyandrosterone as a result of acid hydrolysis of the urine, it is not surprising to find both substances absent. The results show that the administration of ACTH causes a definite increase in the amounts of 11β-hydroxyetiocholanolone (L-P-G-120-I), 11-ketoetiocholanolone (L-P-G-120-III), 11-ketoandrosterone (L-P-G-IV) and in the mixture $\Delta^{9,11}$-dehydroetiocholenolone and etiocholanolone (L-P-G-40-I) excreted.

DISCUSSION

The observations of Burstein (17) and of Ely and Kelly (18) that the guinea pig is the laboratory animal of choice for corticoid excretion studies has been well substantiated in this investigation. Using a relatively small volume of urine we have been able to isolate and quantitate seven corticoids and definitely identify six of these from this source. The reason for the high excretion of corticoids and presumably of the 11-oxygenated 17-ketosteroid is probably due to the fact that the guinea pig has the highest ratio of adrenal weight to body weight, 0.1% in the guinea pig and 0.001% in the human (19). It has been reported by the same authors that the guinea pig excretes about 10 times as much corticoids as the human adult on the basis of body weight. Of the 7 corticoids isolated, C-F-Iac, II-Iac, Vac, VIac and VIII, all steroids with an α-ketol reducing side-chain are excreted in

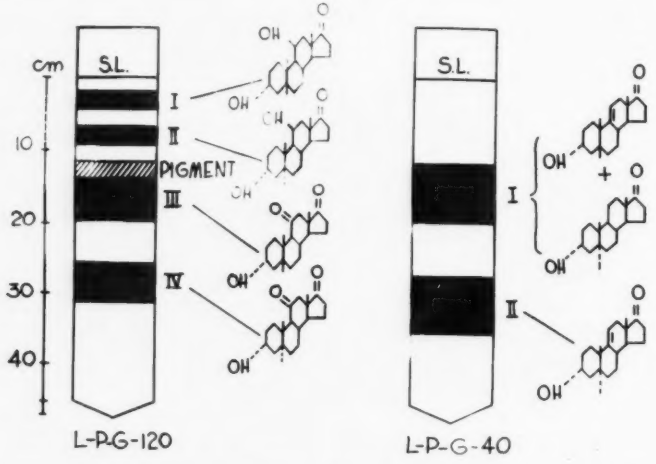

FIG. 2. Diagrammatical representation of paper chromatograms of fractions B_1 run in the ligroin-propylene-glycol system for 120 hours (L-P-G-120) the run-off being subsequently run in the ligroin-propyllene-glycol system for 40 hours (L-P-G-40). S. L. =Starting line.

significantly greater amounts after ACTH administration. Thus, ACTH does not appear to bring about the selective liberation of any one corticoid by the adrenal cortex. However, the possibility exists equally that these substances have one common precursor which, after its liberation by the adrenal gland, is transformed in the process of "inactivation" by extra-adrenal tissues prior to their elimination in the urine.

Accounting for the fact that the corticoids isolated in this study, with the exception of cortisone, were determined as the acetates the total

TABLE 3. QUANTITATIVE ANALYSIS OF 17-KETOSTEROIDS ELUTED FROM LIGROIN PROPYLENE GLYCOL PAPER CHROMATOGRAMS

Zones on chromatograms	Running time, hours	Name of steroid	μg./day control urine	μg./day ACTH urine	Response to ACTH % increase over control
I	120	11β-hydroxy etiocholanolone	22.7	37.3	64
II	120	11β-hydroxy androsterone	—	—	—
III	120	11-ketoetiocholanolone	137.0	287.8	110
IV	120	11-ketoandrosterone	—	21.0	
I	40	Mixture of $\Delta^{9,11}$-dehydroetiocholenolone+ etiocholanolone	75.5	158.0	114
II*	40	$\Delta^{9,11}$-dehydroandrosterone	—	—	—
VII†		6β-11β-dihydroxy Δ^4-androstene-3,17-dione	6.5	4.7	0
			241.7	508.8	

* Both L-P-G-120-II and L-P-G-40-II have been detected in a previous study (3).
† Calculated as the free compound from values in Table 2.

amount of urinary corticoids in both the control and ACTH-treated animals is comparable with those values published for the normal, scorbutic and ACTH-treated guinea pig (2, 20). They are low when compared to the results of Liddle and coworkers (21) who reported a 210 μg./day level (limits 150–400 μg./day) of urinary corticoid in the normal guinea pig and up to a threefold increase in animals injected with 5 I.U. of ACTH per day.

The excretion of the 17-ketosteroids parallels the excretion of corticoids and is higher in the ACTH treated animals than in the normal controls with the exception of 6β,11β-dihydroxy-Δ^4-androstene-3,17-dione. However, it will be seen that the increase in the amount of 17-ketosteroid excreted following ACTH administration is not as great as for the corticoids. In view of the findings of Clayton and Prunty (5) that adrenalectomized guinea pigs do not respond to ACTH and show no change in the amount of urinary 17-ketosteroid excreted in the male or female animal, it can be concluded that the increased output of 17-ketosteroids observed in this investigation is brought about as a result of the response of the adrenal to injected ACTH. Whether the steroids are secreted as such by the adrenal or are conversion products of the corticoids is unknown at the present moment.

This study has also shown that all urinary corticoids isolated thus far are α,β-unsaturated-3-ketone steroids and are present in their organic solvent soluble free form. This is in contradistinction with the well-known fact that in the human most of the corticoids and their reduction products are eliminated in the conjugated form. It can be said, therefore, that conjugation is a necessary sequence to reduction of the 4–5 double bond of ring A. All the urinary 17-ketosteroids with the exception of $6\beta,11\beta$-dihydroxy-Δ^4-androstene-3,17-dione are extracted only following acid hydrolysis, indicating that these compounds are eliminated in their water-soluble conjugated form.

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THE EFFECT OF CORTISONE AND CORTICOTROPIN ON THE RELEASE AND PERIPHERAL METABOLISM OF THYROID HORMONE[1]

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ABSTRACT

Rat thyroid glands were labelled with I^{131} and further uptake blocked with propylthiouracil. The effects of cortisone and corticotropin on I^{131} release rates were studied, each animal being used as its own control.

Corticotropin, 25 or 90 units subcutaneously 12 hourly was without effect. Two and one-half milligrams of cortisone subcutaneously 12 hourly doubled the I^{131} release rate. This increase was also present in hypophysectomized animals. Cortisone did not influence the acceleration of "release rate" produced by thyrotropin. It was concluded that this unexpected effect of cortisone is due in part to a direct action on the thyroid. The hypophysis may also be stimulated to produce excess thyrotropin.

The effects of cortisone and corticotropin, in the same doses, on peripheral degradation of thyroxine, were investigated in rats, using intravenous thyroxine I^{131} and frequent blood sampling over a 72-hour period. Control and experimental groups were used. The mean half-life in the control animals was 19 hours and was not significantly different in either experimental group.

RECENT reviews (1, 2) of work carried out on the effects of cortisone and corticotropin on thyroid function have emphasized the considerable confusion that still exists as to mechanisms, and the many inconsistencies among results from studies otherwise similar. Cortisone is known to reduce iodine accumulation by the thyroid both in man and in the rat but there is no agreement on whether this represents a direct action on the thyroid itself, or interference with thyrotropin secretion or activity. To help solve this problem measurements have been made of gland hormone release rates which are known to be sensitive to changes in circulating thyrotropin. Results in the rat have been conflicting. Albert *et al.* (3) and Perry (4) found the biological decay rate of accumulated I^{131} to be unaltered by corticotropin or cortisone. More recently, Brown-Grant (5) has reported a greatly diminished release rate in rats given large doses of cortisone. These three studies were carried out on animals with unblocked glands. "Release rates" were therefore modified by the effect of cortisone

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on the accumulation of recycled I^{131}, and their interpretation rendered uncertain. Ingbar and Freinkel (2) have demonstrated a reduction by cortisone in rate of release of hormone from human glands blocked by methimazole. The results of similar studies in rats are presented here.

From determinations of protein-bound iodine in serum of patients with myxedema treated with thyroxine (6, 7) and more recently using I^{131}-labelled thyroxine (8), no effect of cortisone or corticotropin on peripheral degradation of thyroxine could be demonstrated. In the rat, Bondy and Hagewood (9) found an increase in serum protein-bound iodine in thyroidectomized animals given thyroxine replacement when treated with cortisone, suggesting a slowing of peripheral breakdown or excretion. A re-investigation of this problem in the rat, using radioactive thyroxine, has also been undertaken.

METHODS

Release rates: 100-gram Sprague-Dawley rats were used in groups of 6 to 10. With one exception the diet fed was Purina Fox Chow, with an iodine content of 1200 μg. per kilogram. In one study Ken-L ration was used containing 230 μg. of iodine per kilogram. Tracer doses of I^{131} were calculated to give a gland content of 2 to 3 microcuries at 40 hours and were injected subcutaneously. With the low-iodine diet the average uptake was 12%, and a 25 μc. dose was used. The higher iodine content of the Purina Fox Chow gave uptakes around 6% and 35 μc. were necessary. Then, when appropriate, further uptake was blocked by a subcutaneous injection of 5 mg. of propylthiouracil in 15% gelatin, followed by incorporation of 0.03% of the drug in the diet.

Cortisone (Merck) was injected subcutaneously 12 hourly as a suspension in 2.5 mg. doses. Corticotropin was used in one study as a commercial preparation (Wilson), 25 units 12 hourly, in another as prepared in this laboratory by purification on oxycellulose in doses of 90 units in arachis oil subcutaneously 12 hourly.

Rates of release of accumulated I^{131} were followed by neck counts using a collimated scintillation counter. Counts were carried out under light ether anaesthesia and prolonged sufficiently to reduce probable error to less than 2%.

With each study, the release period was divided into two parts, a control and an experimental, during each of which over 40 to 60 hours a minimum of 4 counts were made. The appropriate hormone was injected during the experimental period.

Peripheral degradation: 200–250 gram male Sprague-Dawley rats fed on Purina Fox Chow were used. Thyroid block was established as before. Twenty-five microcuries of thyroxine I^{131} in a 0.3 ml. volume containing approximately 0.0017 mg. of carrier were injected by tail vein. Samples of tail tip blood, 0.1 ml., were withdrawn 4 hours later and at approximately 12-hour intervals over the following 72 hours. Samples were counted in a well-type scintillator. Counts were prolonged to give a probable error of less than 2%. Levels of blood radioactivity were expressed as a percentage of the 4-hour value. Three groups of 7 rats were used, either as untreated controls, or with cortisone, 2.5 mg., or corticotropin in oil, 90 units, 12 hourly by subcutaneous injection.

RESULTS

In Table 1 appear values for the release rates during control and experimental periods expressed as percentage release of gland I^{131} per hour. A preliminary experiment (No. 1) was carried out using rats with unblocked

glands. In Experiments 2–5 propylthiouracil was used throughout, commencing at the end of the 40-hour uptake period. This had a pronounced effect in increasing control rates of release of I^{131} from the gland. No significant effect of corticotropin on release could be demonstrated. A consistent and highly significant increase in release rate accompanied the use of cortisone in each of three studies, both in blocked and unblocked glands. Figure 1 and Table 2 present the data from one of these.

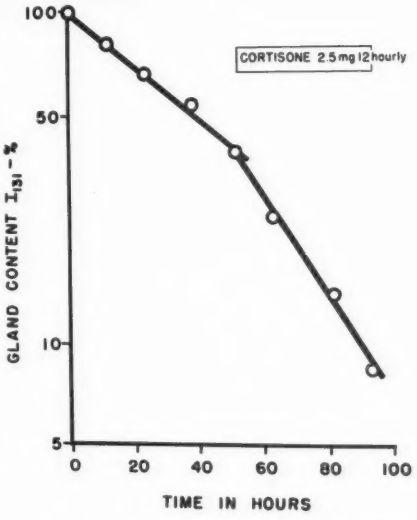

FIG. 1. Results of Experiment 5, showing the increase in release rate of gland I^{131} produced by cortisone.

Three further experiments were carried out in an attempt to elucidate this unexpected finding.

Experiment 6: This was conducted as in Experiments 2–5 except that no hormone injections were given during observation of release over a 120-hour period. The linearity of the semilogarithmic plot was preserved and no significant difference in slope could be demonstrated between the portions of the curve equivalent to control and experimental periods in the previous studies. Percentage release of I^{131} averaged 2.37 per hour (SD $=0.3$). The lower iodine content of the diet fed this group of rats for 5 days prior to study probably accounted for the rather higher release rates than during control periods in Experiments 2–5. Increasing rates of thyrotropin secretion as the result of falling levels of circulating thyroxine following gland blockade could not explain the results in the cortisone studies.

TABLE 1. SHOWING HOURLY PERCENTAGE RELEASE RATES OF GLAND I^{131} BEFORE AND AFTER TREATMENT WITH CORTICOTROPIN AND CORTISONE WITH A STATISTICAL EXPRESSION OF THE SIGNIFICANCE OF DIFFERENCES. FIGURES IN THE COLUMN HEADED "MEAN DIFFERENCE IN RELEASE RATES" REFER TO THE MEAN DIFFERENCES BETWEEN SLOPES OF RELEASE RATE CURVES FOR CONTROL AND EXPERIMENTAL PERIODS IN EACH ANIMAL OF A GROUP. "P" VALUES EXPRESS THE SIGNIFICANCE OF THESE MEANS

Experiment	(No.)	No. of rats	Hourly percentage release rates		Mean difference in release rates	Significance of differences
			Control	Experimental		
Cortisone	(1)	7	0.90 ± 0.18*	1.28 ± 0.28*	0.37 ± 0.12*	$0.01 > p > 0.001$
Corticotropin	(2)	6	1.98 ± 0.70	2.70 ± 0.83	0.56 ± 1.2	$0.3 > p > 0.2$
Corticotropin	(3)	6	2.20 ± 0.40	2.54 ± 0.86	0.35 ± 0.48	$0.2 > p > 0.1$
Cortisone	(4)	6	1.64 ± 0.66	3.39 ± 0.51	1.75 ± 1.73	$0.001 > p$
Cortisone	(5)	6	1.84 ± 0.12	3.59 ± 0.77	1.74 ± 0.73	$0.01 > p > 0.001$

* Standard deviation.

TABLE 2. GIVING DATA FROM EXPERIMENT 5 SHOWN GRAPHICALLY IN FIGURE 1. VALUES FOR GLAND I^{131} ARE AVERAGED FOR 6 RATS WITH STANDARD DEVIATIONS

Time in hours	0	12	23.5	38	52	52	63	81.5	94
Gland I^{131} as percentage of initial content	100	84.1 ± 8.0	67.3 ± 11.7	53.2 ± 4.0	38.1 ± 2.9	Cortisone started	24.5 ± 3.7	14.1 ± 3.0	8.5 ± 3.7

Experiment 7: The effect of cortisone on the action of thyrotropin in the thyroid was investigated. Three groups, each of 6 rats, were used. Glands were labelled and blocked as previously. One control group was observed without further treatment. The other rats each received 1 U.S.P. unit of thyrotropin at 0 and 8 hours after starting prophylthiouracil. In addition, one group received cortisone, 5 mg. 6 hours prior to the first injection of thyrotropin, and 2.5 mg. with the second. The results are shown in Figure 2. Cortisone did not further accelerate the release of I^{131} induced by thyrotropin.

Experiment 8: A study was made of the direct effects of cortisone on the release mechanism in the absence of thyrotropin. Ten rats were given I^{131} tracer doses, hypophysectomized 40 hours later, their glands blocked and

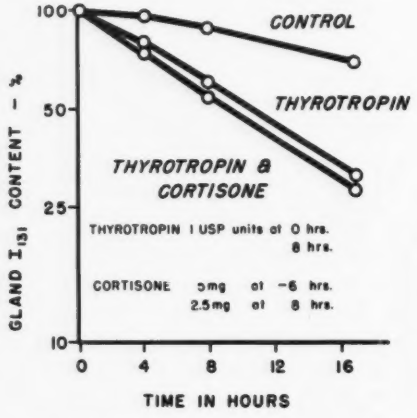

FIG. 2. Results of Experiment 7, showing the failure of cortisone to influence the effect of thyrotropin on release rate.

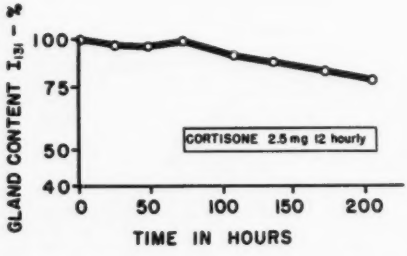

FIG. 3. Results of Experiment 8; the effect of cortisone on release of gland I^{131} in hypophysectomized animals.

neck counts performed at 23 to 36-hour intervals. Virtually no release of I^{131} occurred over a 3-day period. At 74 hours after starting propylthiouracil the glands contained an average of 97% of the tracer I^{131}. Cortisone was started. Subsequent average counts were: 90% at 110 hours, 86.6% at 139 hours, 81.4% at 173 hours, and 77.1% at 207 hours, representing an

immediate and significant increase in rate of I^{131} release ($0.02 > p > 0.01$). The effect is apparent graphically in Figure 3.

In the studies on peripheral degradation of thyroxine, no significant differences could be demonstrated between the experimental and control groups in the rates of fall off of circulating I^{131} (for cortisone $p > 0.5$, for corticotropin $p > 0.4$). Blood levels diminished as a single exponential function with time. Average half-lives were as follows:—control 19.0 hours, SD = 1.4, cortisone 18.2 hours, SD = 2.0, corticotropin 18.8 hours, SD = 1.9.

Discussion: The rapid rate of release of bound thyroidal I^{131} in rats treated with propylthiouracil, the limitation of tracer dose necessary to avoid gland damage, and the advisability of using each animal as its own control made prolonged observations impossible. The data definitely indicated, however, a considerable and highly significant increase in release rate associated with cortisone dosage. The physiological significance of the effect is uncertain, in view of the large doses used and the failure to produce a similar effect with corticotropin. The results are in conflict with those of Perry (4), Albert *et al.* (3), and Brown-Grant (5), as previously described.

The mechanism for this effect is obscure. Experiment 7 revealed no augmenting of thyrotropin's action by cortisone. A direct effect of the steroid on the thyroid cell may play a part, as suggested by Experiment 8, but judging by the limited release rate initiated in that study, is unlikely to be the main factor. A third alternative would be an increase in thyrotropin secretion by the action of cortisone on the hypophysis.

There is some support for such a possibility in the literature Halmi and Barker (10) treated rats with similar doses of cortisone over a 20-day period, and found histological evidence for hypersecretion of thyrotropin, both in pituitary and in the thyroid gland, in the absence of a fall in serum protein-bound iodine. D'Angelo *et al.* (11) found propylthiouracil goitrogenesis in rats to be enhanced by an 18-day course of cortisone, while thyrotropin titres in plasma were increased. Against such a mechanism is the fact that cortisone appears to have no effect on the thyroid/serum concentration gradient for iodide (12, 13), a facet of thyroid function known to be sensitive to variations in circulating thyrotropin.

Acknowledgment

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EFFECT OF RESERPINE ON THE HISTOCHEMISTRY AND CONTENT OF ADRENALINE AND NORADRENALINE IN THE ADRENAL MEDULLA OF THE RAT AND THE MOUSE

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ABSTRACT

Male rats and mice were injected with 1–9 daily doses of reserpine. Adrenaline and noradrenaline contents of the adrenals were determined chemically, and their distributions histochemically, using the chromaffin reaction for both amines and the calcium-formol-induced fluorescence and the iodate reaction for noradrenaline.

In the rat, a single reserpine injection of 0.25–0.5 mg. causes in 24 hours a selective, pronounced loss of noradrenaline from the specific medullary cell islets but does not influence the adrenaline content. The effect is abolished by denervation. Prolonged treatment causes a total loss of both amines.

In the mouse, the contents of both adrenaline and noradrenaline decrease equally after a single injection of 1.0 mg. of reserpine, and a pronounced loss of both amines follows 4–6 daily injections of 0.1 mg.

WIDE clinical use of reserpine has prompted several experimental investigations on this drug. However, to our knowledge only a few studies have been published on the effect of reserpine on the catechol amine content of the adrenal medulla. Holzbauer and Vogt (1) injected cats intraperitoneally or intra-arterially with 0.4–0.7 mg./kg. of reserpine, and observed 24 hours later a decrease in the catechol amine content of the adrenal medulla. The total depletion varied from 28 to 88%, and adrenaline and noradrenaline were equally affected. Cutting of the splanchnic nerve prevented the effect. Carlsson and Hillarp (2) observed in the rabbit a total loss of catechol amines from the adrenal medulla after an intravenous reserpine injection of 5 mg./kg. Denervation was reported not to prevent this effect of reserpine.

It seemed to be of interest to investigate the effect of reserpine on the adrenal medulla of rats and mice. This paper describes such a study, in which attention is paid not only to the amounts but also to the distributions of adrenaline and noradrenaline in the adrenal medulla.

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MATERIALS AND METHODS

Experimental

These experiments involved 65 male rats, aged 2–3 months and weighing 140–180 gm., and 53 male mice about 2 months of age, weighing 15–20 gm. The dosage of reserpine is shown in Tables 1–3 and in Figure 8. The injections were given subcutaneously. The control animals were left untreated. All animals were killed by decapitation with sharp scissors 24 hours after last injection, unless otherwise mentioned.

The left adrenals of 16 rats were denervated by severing the splanchnic nerve immediately below the diaphragm. In addition, a part of the adipose tissue above the left adrenal was removed, avoiding damage to the blood vessels. The reserpine injections were given to the unilaterally denervated animals *c.* 10 days after denervation.

Determination of the catechol amines

The rat adrenals were frozen fresh and cut at 50 μ with a freezing microtome. Some sections were freeze-dried at $-40°$ C, others were used for the study of the fluorescent islets, as will be described later. The medullary part of the freeze-dried sections was removed by microdissection. The pieces were then weighed and applied to the base line of chromatography paper. After chromatographic separation, adrenaline and noradrenaline were quantitatively determined by fluorometry (3).

Mouse adrenals were smeared as a whole directly on the baseline of the chromatography paper and extracted with N/10 hydrochloric acid. Chromatographic separation and determination of the two amines was then made as mentioned above.

The chromaffin reaction

The right adrenal was immersed immediately after killing in a mixture of 1 volume of commercial formalin and 99 volumes of 3.5% potassium dichromate. After 24 hours the mixture was replaced by 10% formalin. Frozen sections were cut at 50 μ, and these were mounted in glycerol.

Histochemical demonstration of noradrenaline

Some fresh-frozen sections from the same glands used for chemical determination were transferred frozen in a mixture containing 1 volume of concentrated formalin, kept over calcium carbonate, and 4 volumes of 2% anhydrous calcium chloride. After fixation for 2–6 hours these sections were mounted in glycerol and examined for fluorescence in ultraviolet light (4).

For the iodate reaction, whole left adrenals were plunged into a saturated reagent of potassium iodate for 24 hours (5), then fixed in 10% formalin and cut at 50 μ with a freezing microtome. The sections were mounted in glycerol and examined in visible transmitted light.

Previous studies suggest that the cells fluorescing after calcium-formol-treatment and those stained brown by iodate are the noradrenaline-containing cells of the adrenal medulla, while a positive chromaffin reaction is given by both adrenaline-containing and noradrenaline-containing parenchymal cells of the medulla.

Statistical analysis

To compare the significance of the observed differences, "Student's" t-test was used.

RESULTS

Effect of reserpine on the rat adrenal medulla

The observations made using the chromaffin reaction and the iodate reaction are summarized in Table 1. In animals killed 4.5 hours after the

reserpine injection, no histochemical changes were detected, even after the enormous dose of 10.0 mg. After 24 hours, doses smaller than 0.1 mg. also had no noticeable effect. A single dose of 0.25 or 0.50 mg. of reserpine caused in 24 hours a disappearance of the iodate-positive cell islets (see also Figs. 1 and 2). In the sections treated for the chromaffin reaction, a brown color was seen after such injection in most of the medullary cells but some cell groups were colorless. The observations suggest that, in the rat, reserpine caused a selective depletion of noradrenaline but had little effect on the adrenaline-secreting cells.

Also in the adrenals taken from animals injected for several days with reserpine the noradrenaline cells were iodate negative (Fig. 3). In addition, a widespread depletion of the chromaffin-positive materials was seen in animals injected for 9 days with a dose of 0.1 mg., indicating a loss of adrenaline. After 8 daily injections of 1.0 mg. of reserpine, all medullary cells were entirely chromaffin negative (*cf.* Figs. 4 and 5). This dose was very toxic, as indicated by the high mortality.

Observations made using the formalin-induced fluorescence for the demonstration of the noradrenaline cells were in agreement with other

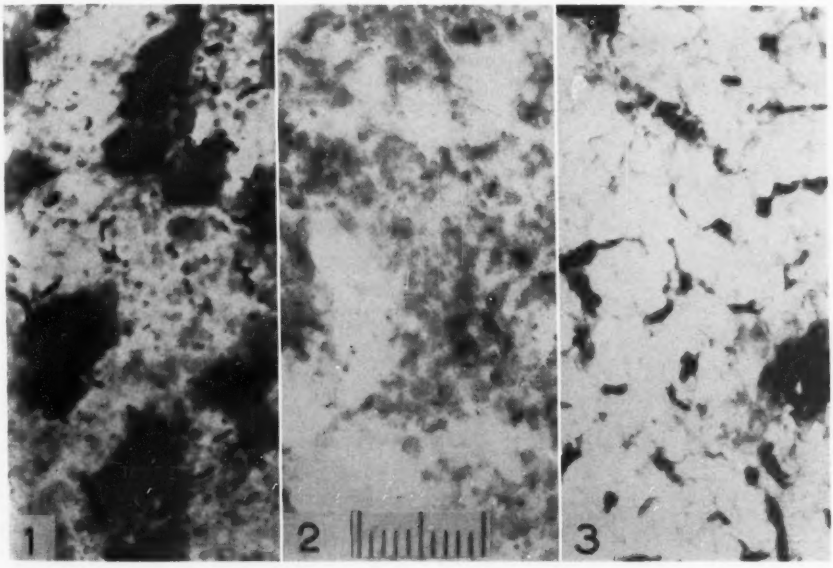

FIG. 1. Adrenal medulla of a control rat. Iodate reaction, no counterstain. Photographed at 4050 Å in transmitted light. The iodate-positive cell islets are clearly visible, the red blood cells black. Magnification ×190.

FIG. 2. Adrenal medulla of a rat injected with 0.25 mg. of reserpine and killed after 24 hours. Technique as in Fig. 1. There is an almost complete loss of iodate-positive materials. Magnification ×190.

FIG. 3. Adrenal medulla of a rat injected with 0.1 mg. of reserpine for 9 days. Technique as in Fig. 1. Only the red blood cells are visible. Magnification ×190.

TABLE 1. EFFECT OF SUBCUTANEOUS RESERPINE INJECTIONS ON THE HISTOCHEMISTRY OF RAT ADRENAL MEDULLA

Dosage (mg.)	Number of animals	Time of killing[1]	Chromaffin reaction (adrenaline+noradrenaline)	Iodate reaction (noradrenaline)
None	12	—	All parenchymal cells +++	Numerous cell islets ++
0.1–10.0	7	4.5	All parenchymal cells +++	Numerous cell islets ++
0.001–0.1	5	24	All parenchymal cells +++	Numerous cell islets ++
0.25–0.50	9	24	Main bulk +++, some cell islets −	All cells −
9×0.1[2]	6	24	Half of medulla −, several cell islets +	All cells −
8×1.0[2]	2/10[3]	24	All cells −	All cells −

[1] Hours after (last) injection.
[2] One injection daily.
[3] Only 2 of the 10 injected rats survived.

data and showed a loss of fluorescence after injection of reserpine (Figs. 6 and 7).

Quantitative determinations of adrenaline and noradrenaline were made of glands from unilaterally denervated rats only. The results are illustrated in Figure 8. It shows that doses of 0.1–0.5 mg. of reserpine, injected daily for 1–3 days, did not cause any difference in the adrenaline concentration

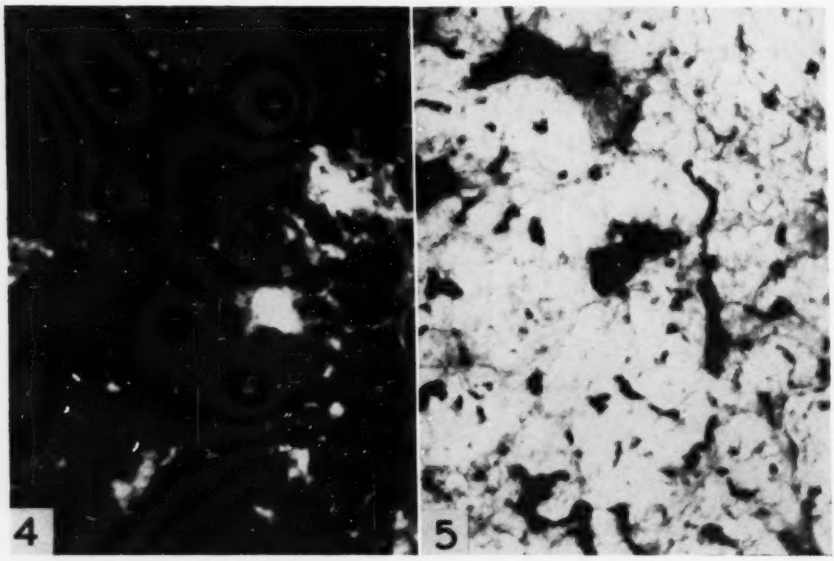

FIG. 4. Adrenal medulla of a control rat. Chromaffin reaction. Photographed at 4050 Å in transmitted light. All parenchymal cells are strongly positive. Magnification ×190.

FIG. 5. Adrenal medulla of a rat injected with 1.0 mg. of reserpine for 8 days. Technique as in Fig. 4. The chromaffin reaction of the parenchymal cells is negative, only red blood cells are visible. Magnification ×190.

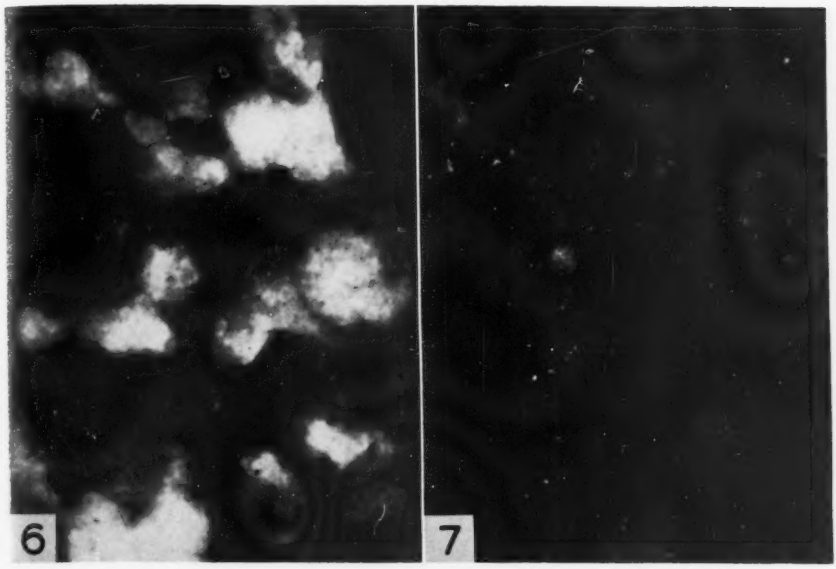

FIG. 6. Adrenal medulla of a normal rat (the same animal as that in Fig. 1). Fluorescence photomicrograph taken after fixation in calcium-formalin solution. The fluorescent cell islets are clearly visible. Red blood cells deep black. Magnification ×190.

FIG. 7. Adrenal medulla of a rat injected with 0.25 mg. of reserpine and killed after 24 hours (the same animal as that in Fig. 2). Technique as in Fig. 6. The fluorescence has almost completely disappeared. Magnification ×190.

of the adrenal medulla of the innervated and the denervated glands. The adrenaline concentrations vary within normal limits in both cases. On the other hand, the noradrenaline concentration was decreased in most intact adrenals of the animals injected with 0.2 or 0.5 mg. of reserpine, although no clear effect was obtained with 3 daily injections of 0.1 mg. of reserpine.

Effect of reserpine on the mouse adrenal medulla

The histochemical data are briefly presented in Table 2. Twenty-four hours after a single injection of 0.04–0.05 mg., some decrease in the intensity of the iodate reaction and in that of the chromaffin reaction of some medullary cell groups were observed, while lower doses were without any observable effect. A single injection of 1.0 mg. caused a distinct loss of chromaffin-positive materials from most medullary cells, and the intensity of the iodate reaction was decreased in many cell islets. However, several strongly iodate-positive and chromaffin-positive cell groups were still present. Because at least some loss of chromaffin-positive material was observed in the majority of medullary cells, of which some normally contain adrenaline, some others noradrenaline, the histochemical observations suggest that reserpine caused a loss of both adrenomedullary catechol amines.

TABLE 2. EFFECT OF SUBCUTANEOUS RESERPINE INJECTIONS ON THE HISTOCHEMISTRY OF MOUSE ADRENAL MEDULLA

Dosage (mg.)	Number of animals	Time of killing[1]	Chromaffin reaction (adrenaline+noradrenaline)	Iodate reaction (noradrenaline)
None	7	—	All parenchymal cells +++	Numerous cell islets ++
0.001–0.02	4	24	All parenchymal cells +++	Numerous cell islets ++
0.04–0.5	7	24	Most parenchymal cells +++, some cell islets + or −	Numerous cell islets + or ++
1.0	5	24	Most cells ++, some cell islets +++	Numerous cell islets + or ++
5×0.1[2]	8	24	Half of medulla −, cell islets + or ++	All cells −
6×0.1	5	24	Half of medulla −, cell islets + or ++	All cells −

[1] Hours after (last) injection.
[2] One injection daily.

Daily injections of 0.1 mg. of reserpine for 5–6 days caused a complete disappearance of the iodate reaction from the medulla. About ½ of the medulla showed a negative chromaffin reaction but the remaining cells, which formed small cell islets in the medulla, were still stained brown, although less so than normal parenchymal cells. These observations seem to show that not only most noradrenaline but also most adrenaline had disappeared from the adrenal.

Results of the two experiments in which quantitative determinations were made are shown in Table 3. In agreement with the histochemical observations, a loss to about one half in both the adrenaline content and the noradrenaline content was found 24 hours after administration of 1 mg. of reserpine. Repeated injections of 0.1 mg. of reserpine also caused a loss of both adrenaline and noradrenaline. The content of noradrenaline was so low that it can be readily understood that no positive iodate reaction was seen in the histochemical experiment in which the same dose was used. The small amount of noradrenaline left was probably mainly present as a precursor in the adrenaline-secreting cells of the medulla, which are more numerous than the noradrenaline-secreting cells and which thus probably contained a concentration of noradrenaline low enough to be below the

TABLE 3. EFFECT OF SUBCUTANEOUS RESERPINE INJECTIONS ON THE CATECHOL AMINE CONTENT OF MOUSE ADRENAL

Dosage	Number of animals	Adrenaline (μg.)		*P*	Noradrenaline (μg.)		*P*
		Mean	SD		Mean	SD	
None	4	4.75	0.03	0.001	2.05	0.02	0.001
1×1.0 mg.	4	2.19	0.06		1.11	0.04	
None	4	8.46	2.36	0.001	4.23	1.05	0.001
4–5×0.1 mg.	5	0.44	0.02		0.64	0.02	

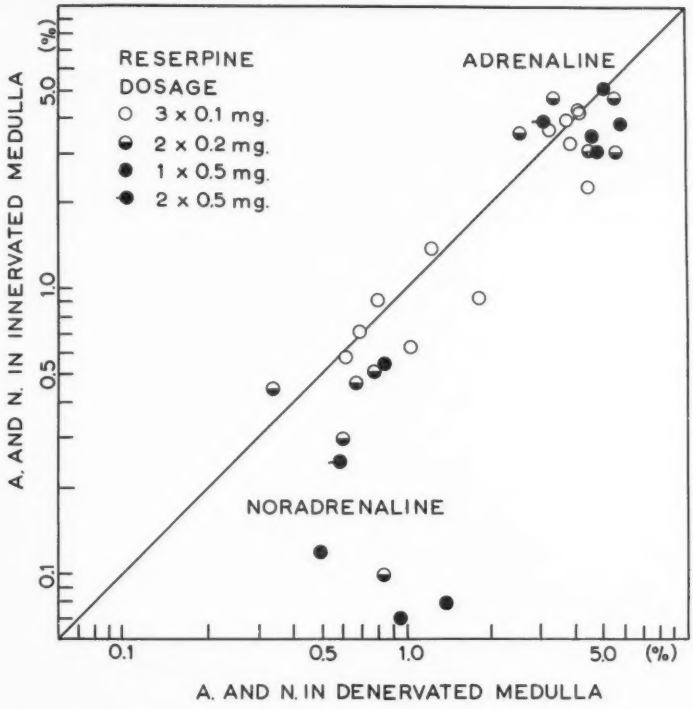

FIG. 8. Comparison of catechol amine concentrations in innervated and in denervated adrenals after reserpine treatment. Concentrations expressed as % of dry medullary tissues. Scales logarithmic. Each circle represents value for one rat.

threshold of the histochemical method. That some chromaffin-positive cell groups were seen in the histochemical studies after repeated injections of 0.1 mg. of reserpine, although chemical determinations indicated an almost complete loss of adrenaline after similar treatment, can be explained either by differences in the responses in the two experiments or by assuming that the small amount of adrenaline was concentrated in relatively few adrenaline-secreting cells.

It should be pointed out that the chemical observations made were statistically highly significant although the number of animals used was small. Therefore, and since the histochemical observations were essentially in agreement with the quantitative determinations, it seems safe to conclude that reserpine causes in the mouse adrenal a loss of both adrenaline and noradrenaline. Some efforts were made to induce a complete depletion of the medullary catechol amines by means of repeated injections of larger reserpine doses. These efforts remained unsuccessful, however, because the mice died before more marked effects on the adrenal catechols was obtained.

DISCUSSION

The present study shows that reserpine causes a loss of adrenomedullary catechols from the adrenal medulla of the rat and the mouse, in agreement with earlier observations on the cat (1) and on the rabbit (2). With regard to the catechol amines affected, the results vary from one species to another. Thus, Holzbauer and Vogt (1) observed in the cat adrenal a loss of both adrenaline and noradrenaline after reserpine injection, and we observed a similar loss in the mouse adrenal. In the rat, on the other hand, a single injection caused a selective loss of noradrenaline, the adrenaline content remaining unaffected. In the rabbit Carlsson and Hillarp (2) found a complete depletion of adrenaline from the adrenal medulla.

These differences are not likely to be due to different methods of treatment or of catechol analysis. This is evident from the recent work by Holzbauer and Vogt (6), of which we became aware through a personal communication by Dr. Vogt after the present study was completed. Holzbauer and Vogt found in agreement with our observations that an intraperitoneal reserpine injection decreases the noradrenaline content of the rat adrenal without influencing the adrenaline content and that reserpine causes a great loss of adrenaline from the rabbit adrenal, as was earlier demonstrated by Carlsson and Hillarp (2).

It thus seems clear that animals of different species respond in a different way to a reserpine injection, as far as the adrenaline and noradrenaline loss is concerned. Why this is so, is difficult to say, particularly since differences were also seen in the responses of the rat, on the one hand, and the cat and the mouse, on the other, although all these animals have separate, specific cell groups for the secretion of noradrenaline, in contrast to the rabbit, in whose adrenal medulla all parenchymal cells are similar containing, and presumably secreting, almost only adrenaline.

Selective depletion of noradrenaline by reserpine from the rat adrenal is a particularly interesting phenomenon. Because the loss was restricted, as was shown by the histochemical observation, to the specific adrenomedullary islets containing noradrenaline, and because denervation prevented at least most of the reserpine-induced loss, it is possible that the noradrenaline cell islets are innervated by fibers from higher centers which are more sensitive to reserpine than the centers from which the fibres innervating the adrenaline cells originate. In this connexion it is of interest to note that Muscholl and Vogt (7) recently reported a selective loss of noradrenaline from the cervical sympathetic ganglion of rabbits injected with reserpine.

Prolonged administration of large doses of reserpine was in our experiments followed by disappearance of all histochemically demonstrable catechols from the rat adrenal but failed to do so in the mouse. Considering that the dosage was large enough to kill 8 out of the 10 rats, this dep'e-

tion may well have been due to a non-specific prolonged stress rather than to a specific effect of reserpine. In the rabbit, the cat and the mouse reserpine apparently causes in a more specific way the depletion of adrenaline.

Species differences exist also with regard to the effect of denervation on the response to reserpine. Thus, in the cat (1) and in the rat (the present work) the denervated adrenal was found to be little, if at all, influenced by reserpine, while in the rabbit Carlsson and Hillarp (2) observed no difference in the response of the denervated adrenal. Holzbauer and Vogt (6), however, found that although reserpine decreases the adrenaline content of the denervated adrenal of the rabbit, the loss is smaller than that in the contralateral intact adrenal. Both in the cat experiments made by Holzbauer and Vogt (1) and in ours with rats all unilaterally denervated animals were injected with reserpine. Therefore, a slight loss of catechols which may have been caused by reserpine even in the denervated glands could not be detected. Such a loss, if existing, was small as compared with the loss from the innervated gland. Therefore, it may safely be concluded that this effect of reserpine is principally, if not entirely, mediated through the splanchnic nerve in the cat and the rat. Why this is not so in the rabbit, remains to be seen.

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THE EFFECT OF ACTH ON THE ASCORBIC ACID CONCENTRATION OF ADRENAL VENOUS PLASMA OF THE RAT[1]

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ABSTRACT

The intravenous administration of 20 mu or 50 mu of ACTH in hypophysectomized rats or "stress" in the intact rat produces an increase in the amount and concentration of ascorbic acid in blood collected from the adrenal vein by direct cannulation. Under the influence of ACTH, the amount of ascorbic acid found in adrenal venous blood could account for that which disappeared from the adrenal, but under "stress" more ascorbic acid disappeared from the adrenal than could be found in the adrenal venous blood. The procedure associated with the cannulation of the adrenal vein and the collection of adrenal venous blood caused a large rise in the circulating levels of ascorbic acid even though the pituitary and adrenals had been effectively removed.

PRELIMINARY data reported earlier by Munson and Briggs (1) showed that adrenocorticotropic hormone (ACTH) increased the concentration of ascorbic acid in adrenal venous blood. The following report presents additional experiments showing that ACTH or "stress" increases adrenal venous ascorbic acid concentration and attempts to evaluate whether or not the drop in adrenal ascorbic acid is equivalent to the rise in adrenal venous ascorbic acid.

METHODS

Adult male albino rats[2] weighing 350–450 gm. were used. Hypophysectomy was performed by the parapharyngeal route 20–24 hours before each experiment. Before cannulation of the adrenal vein the rats were anesthetized with sodium pentobarbital. A long midline incision was made and all obscuring viscera were radically displaced to make the left adrenal vein accessible. A small glass cannula was introduced directly into the adrenal vein. To prevent clotting 600 units of heparin were injected intravenously 3 minutes before cannulation.

The effect of ACTH on adrenal ascorbic acid and on adrenal venous ascorbic acid was measured in the following manner. The right adrenal was removed before injection of ACTH. After the left adrenal vein had been successfully cannulated the dose of ACTH was administered intravenously into the femoral vein. The adrenal vein blood was collected for 20–30 minutes (2–3 ml. of blood). A sample of cardiac blood was taken

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[2] Purchased from Holtzman Company, Madison, Wisconsin as "discards."

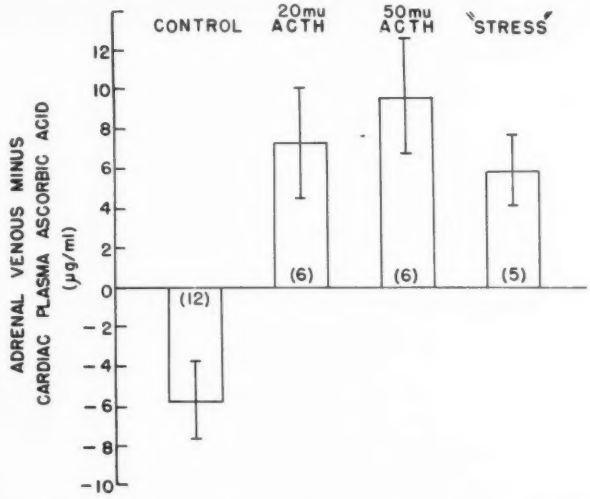

FIG. 1. Effect of ACTH on the difference between adrenal venous and cardiac plasma ascorbic acid concentrations. ( ) indicates number of animals in each group. Vertical bars indicate ± one standard error. The "stressed" rats were not hypophysectomized.

at the end of the experiment and the left adrenal was removed. The decrease in the amount of ascorbic acid in the adrenal was calculated from the difference in ascorbic acid concentration in the right and left adrenals and the weight of the left adrenal.

Ascorbic acid was determined by reduction of indophenol at pH 4.2 (2) or by the dinitrophenylhydrazine reaction (3). A filtrate of the adrenal gland homogenized in 2.5% metaphosphoric acid and filtered with Munktell's 00 filter paper was used to determine adrenal ascorbic acid. A protein free solution of plasma was obtained for estimation of plasma ascorbic acid either with 15% trichloracetic acid (TCA) − 1% metaphosphoric acid or 10% metaphosphoric acid and 95% ethanol at −8° C.

The desired dose of ACTH was dissolved in 1 cc. of a 0.9% NaCl solution in 0.01N HCl (acid saline) and injected slowly into the exposed femoral vein. Similar injections of acid saline were made in the control animals. Either the Provisional U.S.P. Corticotropin Reference Standard or the U.S.P. Corticotropin Reference Standard was used.

RESULTS

Effect of ACTH or Stress on Adrenal Venous Ascorbic Acid

The concentration of adrenal venous ascorbic acid was measured following injection of acid saline (control), 20 mu ACTH, 50 mu ACTH, or following stress[3] and compared with the ascorbic acid concentration of cardiac blood obtained at the conclusion of each experiment. Results are shown in Figure 1. The adrenal venous concentration was significantly ($P < 0.05$) greater than the corresponding cardiac concentration after either ACTH

[3] The cannulation procedure was used to "stress" non-hypophysectomized rats and produced a profound fall in adrenal ascorbic acid (Fig 2).

or stress, confirming our earlier observations and similar observations briefly reported by Slusher and Roberts (4).

Effect of Cannulation Procedure on the Concentration of Circulating Ascorbic Acid

In the experiments summarized in Figure 1 it was found that in the control group the adrenal venous concentration of ascorbic acid was significantly ($P < 0.05$) lower than the cardiac concentration. To test the assumption that ascorbic acid of extraadrenal origin may be entering the blood stream two series of experiments were carried out in hypophysectomized rats. In the first of these experiments the initial ascorbic acid con-

TABLE 1. COMPARISON OF INITIAL AND FINAL CIRCULATING (CARDIAC) BLOOD ASCORBIC ACID CONCENTRATIONS IN HYPOPHYSECTOMIZED RATS

Expt.	Treatment	Circulating ascorbic acid concentration		
		Initial (time 0) μg./ml.	Final (30 minutes) μg./ml.	Per cent increase
1	Laparotomy	8.2	17.8	118.2
2		10.0	13.2	32.0
3		13.5	14.0	3.7
4		13.5	14.8	9.6
5		20.0	23.0	15.0
6		10.5	17.7	68.5
7	Control	22.5	22.0	−2.2
8		7.6	9.7	27.6
9		7.8	8.2	5.1

centration (0.5 ml. sample of cardiac blood) was compared with the final concentration in laparotomized and control animals. The results, Table 1, show that there was a variable increase in ascorbic acid concentration over the 30-minute interval. This is in agreement with recent findings of Allison (5). In the second series of experiments (Table 2) the initial cardiac ascorbic acid concentration was compared with serial samples of adrenal vein blood and a final sample of cardiac blood. It is clear from these experiments that the circulating ascorbic acid concentration was increasing with time even though no ACTH had been given and neither adrenal was in the circulation. Thus the concentration of adrenal venous blood collected during the total experimental period and pooled was lower than the concentration found in a sample of cardiac blood taken at the end of the experiment.

Comparison of the Decrease in Adrenal Ascorbic Acid with the Increase in Adrenal Venous Ascorbic Acid

Figure 2 compares the amount of ascorbic acid lost from the adrenal with the increase in adrenal venous ascorbic acid after stimulating the adrenal with ACTH. The amount of this increase was determined by sub-

TABLE 2. COMPARISON OF SERIAL BLOOD ASCORBIC ACID CONCENTRATIONS IN CARDIAC AND ADRENAL VEIN BLOOD OF HYPOPHYSECTOMIZED RATS

Treatment	Time (minutes)	Cardiac blood ascorbic acid (μg./ml.)	Adrenal vein blood ascorbic acid (μg./ml.)
Control (Acid-saline)	0	10.5	
	5		11.4
	8		12.6
	12		13.0
	18		16.9
	21	17.8	
Control (Acid-saline)	0	11.5	
	15		12.0
	25		12.0
	40		13.9
	51	14.6	
Control (Acid-Saline)	0	16.2	
	10		18.2
	19		19.3
	27		21.7
	40	20.5	
ACTH (50 mu)	0	9.6	
	6	11.4	
	21	12.0	
	26	15.4	
ACTH (50 mu)	0	11.0	
	11	11.6	
	16	11.8	
	21	14.0	
	26	16.4	
	31	16.7	

tracting the average amount of ascorbic acid collected from the adrenal vein of the control group from the average amount of ascorbic acid collected from the adrenal vein of the experimental groups. Correction was made for any differences in the amounts of blood collected from the control and experimental groups. The animals receiving 20 mu of ACTH appeared to secrete more ascorbic acid into the adrenal vein than they lost from the adrenal; this difference was not, however, statistically significant. After injecting 50 mu of ACTH the increase in adrenal venous ascorbic acid was about equivalent to the adrenal depletion observed. In "stressed"[4] nonhypophysectomized animals the amount of ascorbic acid lost from the adrenal was significantly ($P < 0.01$) greater than the increase in the amount of ascorbic acid found in adrenal venous blood.

DISCUSSION

It has been hypothesized by Slusher and Roberts (4) that the fate of ascorbic acid in the ACTH stimulated adrenal gland is secretion into adrenal venous blood. Our results with exogenous ACTH tend to confirm this

[4] In this experiment the right adrenal was not removed until the adrenal vein had been cannulated but was removed before any blood was collected from the vein.

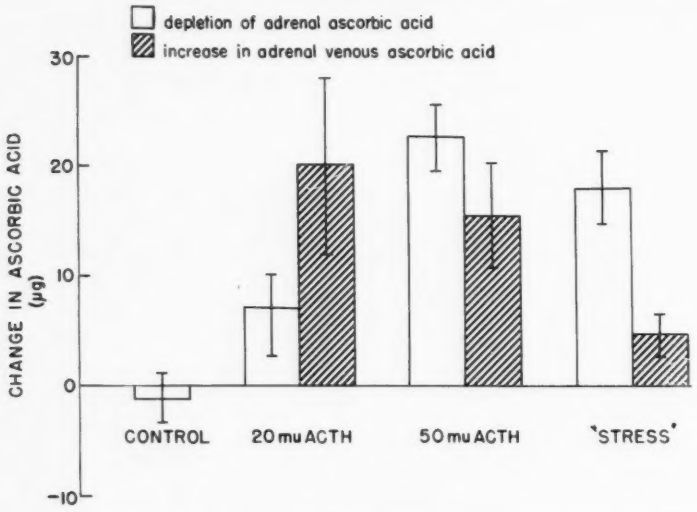

FIG. 2. Comparison of the depletion of adrenal ascorbic acid with the increase in adrenal venous ascorbic acid after ACTH or "stress." Vertical bars indicate ± one standard error. The "stressed" rats were not hypophysectomized.

hypothesis. The results obtained from the normal rats subjected to "stress," however, show that such a hypothesis cannot account for the ascorbic acid depletion observed in these rats. There are certain differences between the experiments with exogenous ACTH and stress which may have contributed to this variation in results. The collection of adrenal venous blood from stressed rats did not begin until about 10 minutes after the beginning of the stress (laparotomy), while with exogenously administered ACTH the collection of adrenal venous blood proceeded from the instant of adrenal stimulation. Further, the stimulation of the adrenal of the stressed rats is continuous throughout the experimental period (6) while estimates of the plasma half-time, from 2 minutes (7) to 5 minutes (8) of exogenously administered ACTH suggests that the plasma level in these experiments fell very rapidly. Such differences in conditions between the two types of experiments allow one to hypothesize that adrenal stimulation initially causes ascorbic acid secretion while continued stimulation leads to a response which is primarily utilization of ascorbic acid.

Acknowledgment

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REVERSAL OF ADRENAL-REGENERATION HYPERTENSION BY AMPHENONE

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ABSTRACT

Rats were operated by unilateral nephrectomy, unilateral adrenalectomy and contralateral adrenal enucleation, they were then given saline to drink. During the process of adrenal regeneration a marked hypertension developed with cardiac and renal hypertrophy. Treatment of adrenal-regeneration hypertensive rats with Amphenone caused a reversal of blood pressure to normotensive limits and decreased the cardiac and renal hypertrophy. Amphenone treatment of rats started two weeks after adrenal-enucleation prevented the anticipated rise in blood pressure. These favourable effects on the blood pressure are accompanied by marked lipid accumulation in the regenerated adrenal gland affecting primarily the fasciculata zone. The administration of Amphenone to normal animals caused no depression in the blood pressure, nor did it affect the hypertension, cardiac hypertrophy, glomerular or arteriolar lesions of adrenalectomized rats given desoxycorticosterone acetate and saline.

We are able to confirm the development of hypertension with adrenal-regeneration in the rat. Our findings support the view that the effect of Amphenone on the blood pressure in adrenal-enucleation hypertension is not a direct effect but is secondary to a depression of the adrenal cortex.

FOR many years a relationship between the adrenal cortex and hypertensive vascular disease in man has been suspected. The laboratory and clinical data which support such a theory has been ably reviewed (1, 2). However, for the most part the evidence is not conclusive, no direct proof exists to implicate the functions of the adrenal cortex in essential hypertension in man and its exact role has remained obscure. Amphenone (1,2-bis (p-aminophenyl) 2-methyl propanone-1), has been shown both in experimental animals (3) and man (4) to have adrenal cortical depressant properties. This study was initiated on the hypothesis that such a compound in providing a degree of control over steroidogenesis by the adrenal, might allow us to throw some light on the role of this gland in experimental hypertension. The demonstration by Skelton that unilaterally nephrectomized adrenal-enucleated rats on a high salt intake develop hypertension during adrenal regeneration (5) appeared to offer a highly suitable experimental method by which this theory could be tested. If in this preparation steroids from the regenerating adrenal cortex are responsible for the hypertension, then depression of this steroidogenesis might be reflected in changes in the blood pressure of the animals. A preliminary report of this study has recently been published (6).

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EXPERIMENTAL

Male hooded rats from our own colony weighing 80–100 gm. were used. They were fed a commercial rat food and given tap water, or following operations, 1% saline to drink. Systolic blood pressures were measured at the ankle by the photoelectric method of Kersten (7). Three readings were taken on each animal prior to operation or treatment and at weekly intervals during the experimental period. Operative techniques used to produce adrenal-regeneration hypertension were similar to those employed by Skelton (5). Anaesthesia was induced by intraperitoneal injection of 30 mg./kg. of sodium pentobarbital. The right kidney and adrenal gland were then removed through an incision in the right paralumbar fossa. The left adrenal was then exposed through the opposite side and a longitudinal incision made in the capsule. Pressure was applied to the gland with small forceps so that the medulla and a major portion of the adrenal cortex was extruded as a solid core which was removed. This technique leaves the adrenal

TABLE 1. BODY AND TISSUE WEIGHTS

Group	Thymus[1]	Thyroid	Adrenal	Pituitary	Heart	Liver	Kidney	Body weight (gm.)
Normal Untreated	115± 8[2]	6±1	13±1	3±1	335± 8	4.24±0.1	684±24	249.4± 5.6
Adrenal-Enucleated Untreated	148±11	7±1	12±1	3±1	425±15*	4.59±0.3	605±34	233 ±10.9
Normal Amphenone Treated	78± 4*	24±1*	23±1*	5±1	306±22	7.95±0.6*	680±37	215 ± 9.8
Adrenal-Enucleated Amphenone at 5 weeks	64± 8*	16±2*	12±1	5±1	313±23	7.10±0.8*	451±44	168 ±15.3
Adrenal-Enucleated Amphenone at 3 weeks	97±17	17±2*	10±2	5±1	312±13	13.4 ± .4*	510±29	172 ±12.3
Adrenal-Enucleated Amphenone at 2 weeks	84±11	15±1*	12±2	5±1	326±27	7.70±0.2*	478±40	174 ± 6.6
Adrenalectomized, DOCA and Amphenone Treated	81±12	19±3*	—	4±1	437±40*	8.22±0.1*	590±60	233 ±14.0
Adrenal-Enucleated Estrogen Treated	123± 9	8±1	14±2	4±1	432±48*	4.94±0.2	681±34	178 ±12.0

[1] Tissue weights are expressed in mg., or in the case of the liver as gm., per 100 gm. of body weight.
[2] Mean value and standard error.
* Significant at the 95% level of confidence compared to normal untreated controls.

capsule and adherent particles of glomerulosa tissue intact to serve as a nucleus for regeneration. The animals in which hypertension was induced by treatment with desoxycorticosterone acetate (DCA) were operated upon by the same procedure except that both adrenals were entirely removed. These animals were then given daily subcutaneous injections of 1 mg. of DCA in propylene glycol.

At the times noted in each Figure, Amphenone treatment was started at a dose of 200 mg./kg. The material was dissolved in distilled water and given in a volume of 1 cc. by stomach tube. Premarin (R), a mixture of water soluble estrogen conjugates of equine origin, was given orally in distilled water. The daily dose was equivalent in estrogenic potency to 1 mg./kg. of sodium estrone sulfate. During the study the animals were weighed each week. The final body weights which accurately reflect changes during the study are given in Table 1. Upon completion of the experiments, the animals were sacrificed using chloroform, their organs were dissected free of fat, weighed and fixed in 10% Formalin or Bouin's solution. Sections of adrenal, pancreas, pituitary, testis, thymus and thyroid, and of brain, heart, intestine, kidney, liver, lung, mesentery, spleen and stomach were embedded in paraffin and stained with haematoxylin-eosin and Masson's Trichrome stain. The adrenal, kidney and liver were also examined in frozen sections stained with Sudan III.

RESULTS AND DISCUSSION

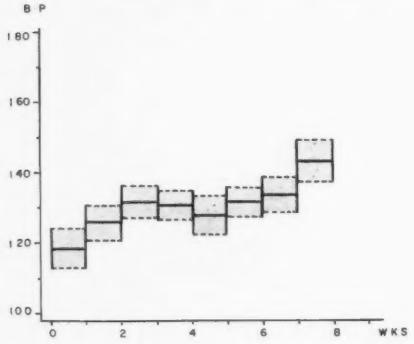
Blood Pressures

As a measure of reliability of the method of testing the blood pressure, an analysis of variance was done on measurements of blood pressure from

5 normotensive animals chosen at random from the control group. A 4-week period was used and the analysis done on a total of 60 blood pressure measurements. It will be noted, Table 2, that no significant difference was seen between replicate readings and that the Index of Precision was high. Significant differences were noted between the blood pressure of the different animals in the group and between measurements taken at weekly intervals. Figure 1 shows the average blood pressure of a group of normal rats during an 8-week period, as the animals become mature a gradual increase in mean blood pressure is seen.

Figure 2 illustrates the typical hypertensive response which is coincident with adrenal regeneration in the rat. Our findings in this respect are in close agreement with the original description of adrenal-regeneration hypertension by Skelton (5). When normal intact rats are treated with Amphenone for a period of seven weeks as in Figure 3, no depressor re-

In these graphs the solid horizontal lines represent the average weekly systolic blood pressure and the shaded areas the 95% confidence limits.



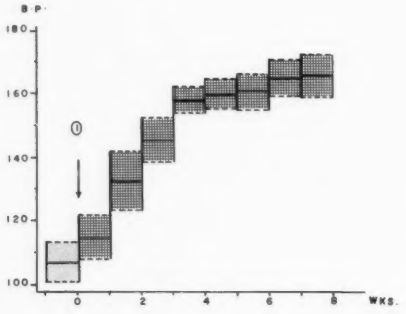
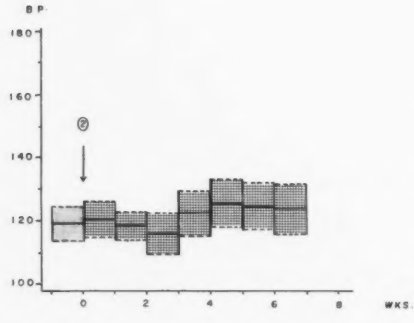


FIG. 1 (left). Normal intact control rats, 15 animals.
FIG. 2 (right). Adrenal enucleation at 1 without treatment, 15 animals.



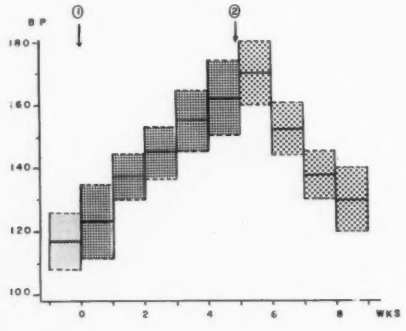
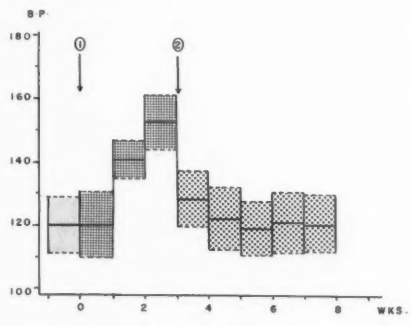


FIG. 3 (left). Normal rats treated at 2 with Amphenone, 15 animals.
FIG. 4 (right). Adrenal enucleation at 1, Amphenone treatment started at 2, 8 animals.

sponse is seen and there are no significant differences between the blood pressures of these animals and untreated controls. If rats, which are hypertensive following adrenal enucleation, are treated with Amphenone 5 weeks after operation (Fig. 4) a progressive decrease in blood pressure is seen until at 9 weeks the average blood pressure of these animals is within normotensive limits. If treatment with Amphenone is instituted three weeks after adrenal enucleation the return of the blood pressure of these animals to normal is more prompt and with continuation of this therapy the blood pressures remain in the normal range. Figure 6 shows the results of commencing Amphenone therapy at two weeks after adrenal enucleation. Mean blood pressures of the group are within normal limits during the entire experimental period.

If Amphenone in these studies was acting through central reflex mechanisms responsible for controlling blood pressure, or by interference with peripheral actions of hormones from the regenerating adrenal cortex, then a depressor response such as that seen in adrenal-regeneration hyperten-



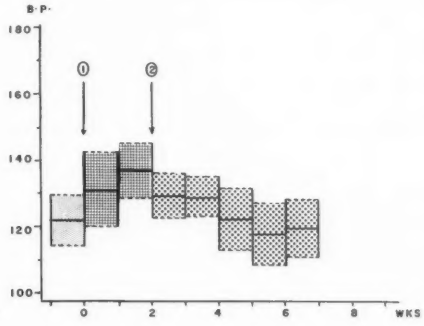
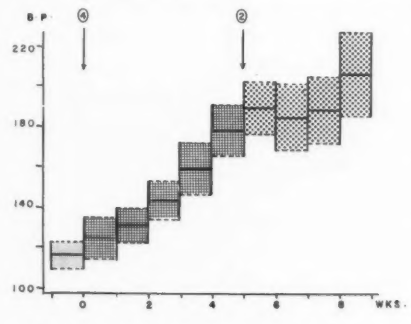


FIG. 5 (left). Adrenal enucleation at 1, Amphenone treatment started at 2, 7 animals.
FIG. 6 (right). Adrenal enucleation at 1, Amphenone treatment started at 2, 9 animals.



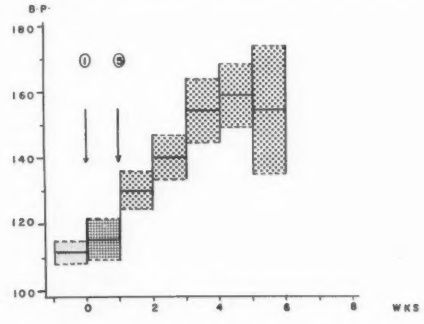


FIG. 7 (left). Bilateral adrenalectomy and DOCA administration at 4, Amphenone treatment started at 2, 12 animals.
FIG. 8 (right). Adrenal enucleation at 1, Premarin administration started at 5, 10 animals.

sion would be expected in hypertension produced in the absence of the adrenal by the administration of DCA. The results of such a study, as shown in Figure 7, demonstrate that Amphenone treatment over a four week period had no effect on DCA hypertension. Thus Amphenone is unlike hypotensive agents such as Rauwolfia or Veratrum derivatives and ganglionic blocking agents in its mechanism of action, since these latter therapeutic agents have been shown to exert a depressor effect on DCA hypertension in the rat (8). These experiments, we feel, offer strong evidence that the hypotensive action of Amphenone in the adrenal enucleated rat is secondary to a depressant effect on the functions of the adrenal cortex.

Amphenone has been reported to possess a weak estrogenic action (3) and Vogt has reported the adrenal cortical depressant properties of a synthetic estrogen (9). We have used a natural estrogen to test whether estrogenic activity per se could be implicated in the action of Amphenone on blood pressure. Figure 8 contains the results of an experiment in which a high dose of an orally active estrogen preparation was administered to rats after adrenal enucleation without influencing the resultant hypertensive response. Thus it appears unlikely that estrogenic activity is involved in the hypotensive mechanism of Amphenone.

Body and Tissue Weights

The average body and tissue weights of the animals from each group at autopsy are contained in Table 1. Normal rats treated with Amphenone show the enlargement of the thyroid, adrenal glands and the liver which have previously been reported (3). The increase in liver and thyroid weight is seen in all groups treated with Amphenone. It will be noted that adrenal regeneration has occurred in all groups after adrenal enucleation irrespective of the treatment given. The changes in heart size noted at autopsy are in agreement with the blood pressure findings. The untreated adrenal-enucleated group, or a similar group treated with Premarin, and the adrenalectomized animals given DCA and Amphenone have the cardiac hypertrophy which was to be anticipated on the basis of their unrelieved hypertension. However, the mean heart size of adrenal-enucleated groups treated with Amphenone is within the range of the normal untreated controls.

After unilateral nephrectomy and adrenal enucleation, hypertrophy of the remaining kidney ensues until at eight weeks the single kidney from such an animal is almost equal to the combined weight of the kidneys from a normal control. Amphenone has little effect on the renal weight of normal animals, but appears to depress the renal hypertrophy which occurs after adrenal enucleation and unilateral nephrectomy. This inhibition of renal hypertrophy is absent in the animals given DCA following adrenalectomy and then treated with Amphenone. The failure of Amphenone to influence the renal weight of normal animals or prevent the renal hyper-

TABLE 2. ANALYSIS OF VARIANCE OF BLOOD PRESSURE ON NORMAL ANIMALS, THE BLOOD PRESSURE HAVING BEEN TAKEN ON FIVE ANIMALS IN TRIPLICATE AND FOR FOUR CONSECUTIVE WEEKS

Animal	1st week	2nd week	3rd week	4th week	Average
1	130	129	130	128	129.4
	128	130	131	128	129.4
	126	127	129	130	128.0
Average	128.0	128.7	130.0	128.7	128.8
2	128	130	112	111	120.2
	130	131	114	110	121.2
	130	128	114	110	120.3
Average	129.3	129.7	113.3	110.3	120.7
3	136	137	128	128	132.3
	138	136	130	128	133.0
	138	137	130	129	133.9
Average	137.3	136.7	129.3	128.3	132.9
4	140	140	140	138	139.5
	140	142	140	137	139.7
	142	142	139	137	140.0
Average	140.7	141.3	139.7	137.3	139.8
5	133	129	128	128	129.5
	128	129	128	130	128.8
	132	130	129	130	130.3
Average	131.0	129.3	128.3	129.3	129.5
Grand Averages	133.3	133.1	128.1	126.8	130.3

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio	Significance
Between Animals (5)	2,301	4	575	38	High Probability $< .01$
Between Weeks (4)	506	3	169	11	High Probability $< .01$
Between Replicates (3)	1	2	0.5	<1	None
Error	749	50	15*	1	—
Total	3,557	59			

* Estimate of Error $= \pm\sqrt{15} = \pm 3.9\%$.
Index of Precision $= 96\%$.

trophy in the DCA group would suggest that the absence of compensatory hypertrophy in the adrenal regeneration groups cannot be attributed to a direct action of Amphenone. We feel that the lower renal weights like the lower cardiac weights are a reflection of the diminished blood pressure in the adrenal regeneration groups treated with Amphenone.

A thymolytic activity for Amphenone has been reported by Hertz (3). Our data in general confirm this finding as the thymic weights of groups treated with Amphenone are lower than those of control animals. However, in only two groups is the difference statistically significant because of wide variation between individual animals. Clarification of this point must await further experiments in larger groups of animals.

The body weights of the animals at autopsy are difficult to compare since not all the studies were of the same duration. The animals treated with Amphenone or with the comparatively high dose of estrogen showed a depression in growth which is reflected in the lower weights of these rats at autopsy.

HISTOPATHOLOGICAL EXAMINATION

The discussion of the histopathology of animals from these studies will be limited to those of three groups. No differences were noted in the histological appearance of tissues from adrenal-enucleated hypertensive rats which had received Amphenone for either four or five weeks. For this reason these animals are discussed as one group.

Group 1. Intact animals treated with Amphenone

Treatment of normal animals with Amphenone caused histopathological changes in the adrenal cortex, thyroid, pituitary, and liver; other tissues were normal.

Adrenal. The adrenal cortex was markedly widened, an increase in the width of the fasciculata, and to a lesser extent of the reticularis zone, being responsible. The cells of all zones were filled with lipids, with the exception of a narrow lipid free zone between the zona glomerulosa and the zona fasciculata. Lipid accumulation in cortical cells was generally even and the nuclei were central; however, in the outer half of the zona fasciculata occasional cells showed a picture of fatty metaplasia with the nucleus pushed to the side. These changes may be observed in a comparison of Figures 9 and 10.

Pituitary. The predominant change in the pituitary was enlargement and vacuolization of basophilic cells. This vacuolization of the basophils occurred in approximately fifty percent of cells of the anterior pituitary in some animals and in only a scattered few cells in others. These changes are now being studied by more specific staining methods.

Thyroid. The enlarged thyroid glands were composed of dilated acini with many papillary infoldings which were almost devoid of colloid. Occasionally the epithelium which had undergone hyperplasia narrowed or occluded the lumina of the acini. The epithelium was composed of tall columnar cells with nuclei arranged close to the basement membrane.

Liver. The architecture of this organ was well maintained, but with dilatation of the central veins and centrolobular congestion. In the periphery of the liver lobules the liver cells showed a picture of fatty infiltration (Fig. 11). The nuclear staining was well preserved and many cells contained mitotic figures or two nuclei.

Group 2. Adrenal-regeneration hypertensive animals without treatment

Adrenal. The adrenal cortices of all animals had undergone regeneration with clear zonal differentiation. The zona glomerulosa was thin and com-

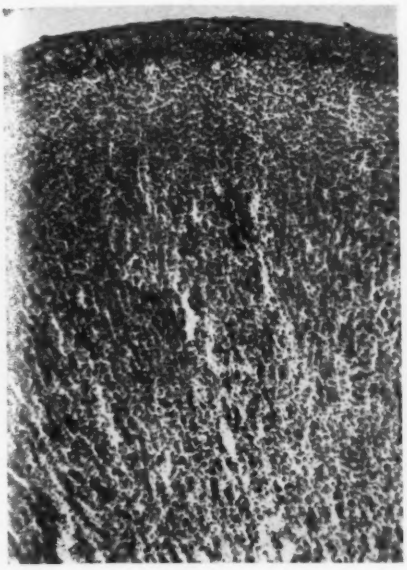


FIG. 9

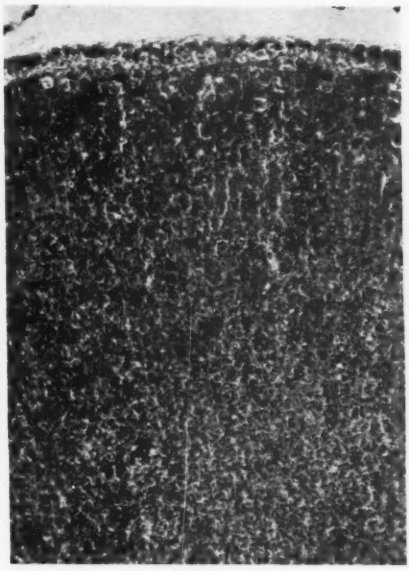


FIG. 10

FIG. 9. Normal adrenal cortex. Sudan III and Haematoxylin, ×125.

FIG. 10. Adrenal cortex from intact animal treated for 7 weeks with Amphenone. Heavy lipid accumulation can be seen in all zones with a lipid free layer of cells between the glumerulosa and fasciculata zones. Sudan III and Haematoxylin ×125.

posed of three or four layers of flattened, darkly stained cells in which some mitotic figures could be seen. The zona fasciculata was markedly thickened with venous engorgement between the cell columns. The zona reticularis was normal. The lipid content of all layers was depleted. The middle of the regenerated adrenals contained a fibrous hyaline mass in some areas of which calcium deposition was noted.

Kidneys. The kidneys of these operated control animals failed to show any evidence of the arteriolar lesions described by Skelton (5). In two animals the glomeruli were enlarged with thickened oedematous basement membranes as shown in Figure 12, the capillary tufts were anaemic and occasional glomeruli were hyalinized. In some areas of the kidney, subcapsular microscopic scars were seen with round cell infiltration. The cells of the first convoluted tubules were enlarged and contained large vacuoles and in the lumen of some convoluted and collecting tubules there was hyaline cylinder formation.

Thyroid. The thyroids in most animals were normal although two showed some degree of atrophy with small acini having flattened epithelial linings.

Morphological lesions were absent in all other organs and no alterations of the vascular tree could be detected.

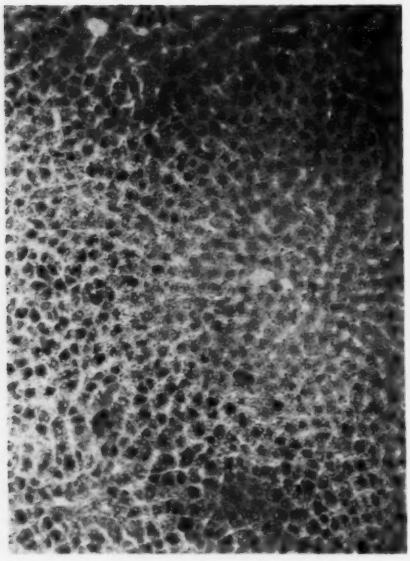


FIG. 11

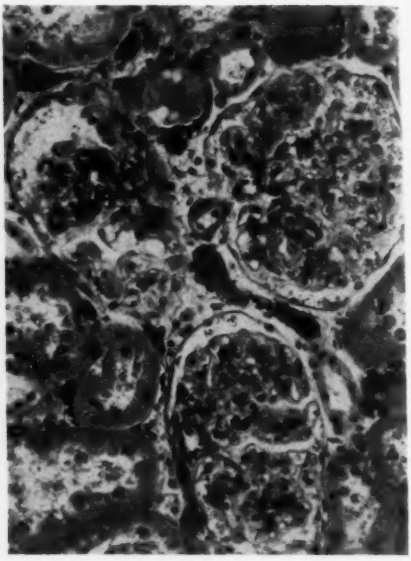


FIG. 12

FIG. 11. Liver of intact rat treated with Amphenone for 7 weeks, perilobular lipid infiltration is apparent. Sudan III and Haematoxylin ×125.

FIG. 12. Kidney from adrenal-regeneration hypertensive rat 8 weeks after adrenal enucleation. The thickened edematous basement membrane and mild hyaline changes were limited to 2 animals. Frank glomerulonecrosis was absent. Masson's Trichrome ×225.

Group 3. Adrenal-regeneration hypertensive animals treated with Amphenone

Adrenal. Amphenone treatment as shown by adrenal weights and histology did not hinder regeneration of the enucleated adrenal. However, there were definite modifications of the histological architecture of cortical cells. In contrast to the animals of Group 2 there was no distinct border between the different cortical zones. The regenerated cortex was uniformly composed of clear, vacuolated cells, the cytoplasm of which—as shown by Sudan III staining—was densely filled with lipid material. The regenerated cells of the adrenal cortex thus closely resemble those of Group 1, Amphenone treatment in each case resulting in a marked increase in lipid content. The connective tissue proliferation and calcification in the middle of the regenerating adrenal was not altered by Amphenone treatment. This poor cortical cell differentiation and uniform lipid accumulation is shown in Figure 13.

The pituitary and thyroid glands of the animals from this group showed lesions which were identical to those seen in normal animals treated with

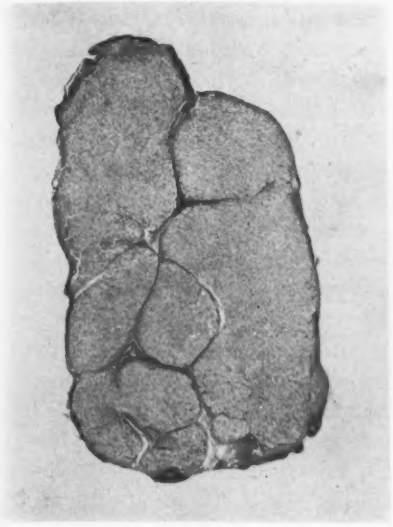


FIG. 13

FIG. 14

FIG. 13. Regenerated adrenal cortical tissue 8 weeks after adrenal enucleation. Amphenone was administered during the last 5 weeks of this period. Marked nodularity is present. The uniform pale appearance can be attributed to the dissolution of lipids. When compared with Fig. 14 the absence of zonal differentiation may be seen. Haematoxylin and eosin ×20.

FIG. 14. Regenerated adrenal cortical tissue 8 weeks after adrenal enucleation. Differentiation of the cortical zones can be distinguished. Haematoxylin and eosin ×20.

Amphenone as in Group 1. The histological picture of the kidney was normal, none of the animals showed glomerular or arteriolar lesions.

These studies have confirmed the findings of Skelton that hypertension develops in unilaterally nephrectomized adrenal-enucleated rats on a high salt intake during the process of adrenal regeneration (5). Amphenone treatment of these hypertensive animals caused a reversal of established hypertension. If treatment was started soon after adrenal enucleation, it prevented the development of hypertension. We have failed to produce vascular lesions following adrenal regeneration and only two animals showed slight glomerular lesions. Skelton has emphasized the greater sensitivity of younger rats to the development of hypertension after adrenal regeneration (10). Our animals had body weights at operation of approximately 100 gm., while those of Skelton weighed an average of 62 gm. This difference we feel might explain the absence of glomerular and arterial lesions in our studies.

Amphenone treatment of either normal rats or adrenal-enucleated rats caused marked increases in the stored lipid of adrenal cortical cells. This phenomenon has been described in normal animals by Hertz *et al.* (3), and

interpreted as an indication of depression of adrenal cortical function. Rosenfeld and Bascom (11) demonstrated that Amphenone could inhibit steroidogenesis by the adrenal gland and Hertz *et al.* have evidence of a depression of steroid biosynthesis following the administration of Amphenone to man (4). This adrenal cortical depressant activity of Amphenone is, we feel, primarily responsible for its effect on the hypertension of adrenal regeneration. The failure of Amphenone to influence hypertension induced in adrenalectomized animals by the administration of DCA is in agreement with this thesis.

These findings also add weight to the supposition that adrenal regeneration hypertension is in fact caused by increased or imbalanced secretion of adrenal cortical hormones by the regenerating tissue, since, as shown in this study, the administration of a compound with known adrenal cortical depressant properties causes a prompt reversal of the hypertension.

The development of agents such as Amphenone with adrenal cortical depressant properties yet free of the side effects and toxicity which have been described for this compound, would provide useful diagnostic agents and enable the clinician to better evaluate the role of the adrenal gland in hypertensive vascular disease in man. Their therapeutic use may also be important.

Acknowledgments

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NON-STEROID OVARIAN HORMONES: OBSERVATIONS ON THE MECHANISM OF THE ACQUISITION OF RELAXIN RESISTANCE IN GUINEA PIGS[1]

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ABSTRACT

A study was made of the mechanism by which guinea pigs used for repeated relaxin assays develop resistance to its action. The decline in relaxin sensitivity is not due to prolonged estrogen treatment, nor to aging of the animals, since it was observed in those receiving both estrogen and relaxin, but not in those receiving estrogen alone for a like period of time. The responses of guinea pigs to relaxin from pregnant rabbit serum and to relaxin from sows' ovaries were compared before and after repeated injections of the latter. It was found that a marked (15–20 fold) diminution in sensitivity to sow ovary relaxin is accompanied by a much smaller (2–4 fold) decrease in responsiveness to relaxin from pregnant rabbit serum. It is postulated that the development of the refractory state is due to the formation of an antihormone of limited species specificity.

IT WAS shown recently (1) that guinea pigs used for relaxin assay gradually developed resistance to its action. In the procedure routinely used, guinea pigs are given one microgram of estradiol daily for four days: the relaxin unknown is injected on the morning of the fifth day and the extent of relaxation determined by palpation six hours later. The sequence of estrogen and relaxin injections is repeated on alternate weeks. Under these conditions, maximum sensitivity to relaxin is observed two to four weeks after beginning estrogen treatment. Thereafter, the average sensitivity of a group of guinea pigs remains essentially constant for two months or longer, after which a more or less abrupt decline in responsiveness sets in.

In a typical instance, a relaxin preparation of which 0.050 mg. caused well-defined symphyseal relaxation in each of a group of guinea pigs when they were maximally sensitive was ineffective five months later in doses as high as 0.40 mg. It was also shown that the increased resistance to relaxin was not a consequence of an increased estrogen requirement; animals found to be unresponsive to relaxin remained so even when the amount of estradiol was increased twenty-fold.

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This paper describes some experiments which were undertaken to elucidate further the mechanism of loss of sensitivity. The results indicate that resistance to the action of relaxin under the conditions described above probably has an immunological basis.

EXPERIMENTAL

Guinea pigs were received into the assay colony as immature, virgin females weighing between 275 and 325 gm. Both ovariectomized and intact animals were used. One twentieth ml. of a solution of estradiol-17-β in sesame oil (0.020 mg./ml.) was injected daily for 4 days. Relaxin assays were performed as previously described (2) on the 5th day, the results being expressed in terms of the 'Total Response' (T.R.)[2] of each group of 8–12 animals.

Two relaxin preparations were used. One, designated 126–1, was isolated from acetone-dried pregnant sow ovarian powder[3] by the method of Frieden and Hisaw (2) and contained 40–60 GPU/mg. The other preparation, referred to as 64–1, was isolated from rabbit blood collected between the 25th and 28th day of gestation. The method of Albert and Money (3) was used to concentrate the serum; the concentrate was stored as a dry powder after lyophilization. Its potency in repeated assays averaged 4 GPU/mg. in 1952 and 3 GPU/mg. in 1957.

RESULTS

Two types of experiments were performed. For the first, twenty guinea pigs were ovariectomized and divided into two groups (A and B). Both groups were primed with estradiol every other week for the duration of the experiment (4 months). Each animal of group A also received 0.050–0.100 mg. of preparation 126-1 every other week, and the responses to these injections noted. When the response of the animals in group A had markedly diminished, a comparison was made of the response of the two groups to the same relaxin preparation.

The results are summarized in Table 1. It may be observed that $3\frac{1}{2}$ months after the start of the experiment, 0.20 mg. of 126-1 was less effective in group A than was 0.050 mg. three months earlier. The response of group B to 0.050 mg. 126-1, on the other hand, was undiminished. These data indicate that the refractory state develops as a result of repeated relaxin injections, and is not due to prolonged estrogen treatment; nor is it a normal accompaniment of aging in guinea pigs.

The second series of experiments was designed to explore further the role of immune phenomena in resistance to relaxin. Earlier studies had shown that although precipitates were obtained when solutions of relaxin were mixed with serum obtained from resistant animals, the relaxin content of the supernatant solution was not thereby diminished. Since this

[2] A "Total Response" of 50 or less represents little or no activity, while values of 150 or more indicate marked relaxation. A T.R. of 120 corresponds to 1 Guinea Pig Unit (GPU) of relaxin.

[3] A generous supply of this powder was made available to us by the Armour Laboratories.

experiment involved only a single "antiserum":"antigen" ratio, and since the possibility exists that any antigen-antibody complex which might be formed might remain in solution, these experiments were judged to be inconclusive. An alternative approach to the problem was therefore devised. If the resistance observed in guinea pigs given repeated injections of relaxin from ovaries of pregnant sows is due to formation of a circulating anti-hormone, such animals would be expected to be relatively more responsive to relaxin from another species of animal (e.g., the relaxin of

TABLE 1. EFFECTS OF REPEATED ESTROGEN AND RELAXIN INJECTIONS UPON SENSITIVITY TO RELAXIN

Date	Material injected	"Total Response"	
		Group A	Group B*
March, 1956	126-1, 0.050 mg.	180	155
	0.100 mg.	250	—*
April, 1956	126-1, 0.050 mg.	230	—
May, 1956	126-1, 0.050 mg.	32	—
	0.100 mg.	145	—*
July, 1956	126-1, 0.050 mg.	—	220
	0.100 mg.	50	280
	0.200 mg.	130	—

* Group B received no relaxin between March and July.

pregnant rabbit serum). This phenomenon would be signalled by a significant decrease in the ratio of activities of the two preparations described above compared to that found in maximally sensitive animals.

Accordingly, observations were made, in several groups of guinea pigs, of the amounts of 126-1 (sow ovary relaxin) and 64-1 (pregnant rabbit serum relaxin) required to produce relaxation after the animals in these groups had become refractory to sow ovary preparations. These experiments indicated that although the effectiveness of both preparations was diminished, the apparent decrease in activity of 126-1 was proportionately much greater; the ratio of activities of the two preparations in refractory animals was approximately 4:1, compared to the expected value of about 15:1 based upon assays in sensitive animals.

More extensive data were obtained in two groups of guinea pigs. Group XX had been introduced into the assay colony in early October, 1956. In the middle of January, 1957, after the response to standard sow ovary preparations had sensibly diminished, comparisons were made of the activities of the two preparations. The data are shown in Figure 1, in which a dose:T.R. curve is plotted for each preparation. It may be seen that the apparent activity ratio (126-1:64-1) is less than 2.

Group XXI received its first priming course of estrogen early in December, 1956. After preliminary injections of relaxin had established the sensi-

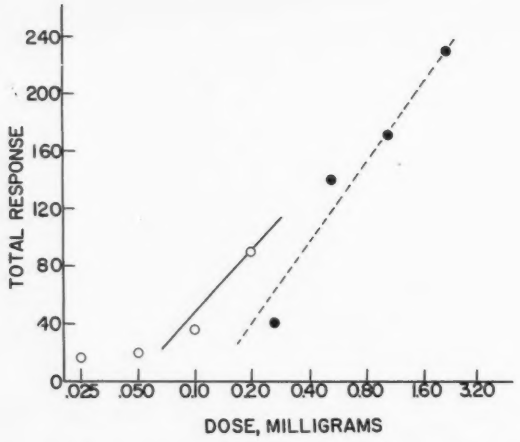

FIG. 1. Dose-Total Response curves for preparations 126–1 and 64–1, using guinea pigs of group XX. Open circles, solid line: 126–1: extrapolated (to T. R. = 120) potency is 3 GPU/mg. Filled circles, broken line: 64–1; interpolated potency is 1.8 GPU/mg.

tivity of this group of animals, a comparison was made of its response to 64-1 and 126-1. Five months later (various sow ovary preparations having been injected in the interim), the comparison was repeated. The data are summarized in Figure 2.

It is clear that a profound change in activity ratio has occurred during the five months encompassed by this experiment. While the data for preparation 126-1 obtained in May, 1957 do not permit an accurate estimate of

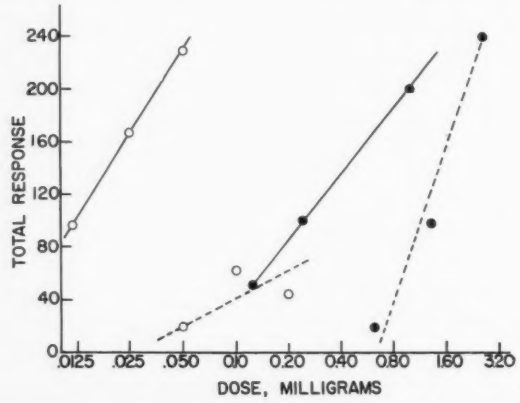

FIG. 2. Dose-Total Response curves for preparations 126–1 and 64–1, using guinea pigs of group XXI. Open circles: 126–1; filled circles: 64–1. Solid lines: data of January, 1957; broken lines: data of May, 1957. Potency estimates: January, 1957—126–1 = 60 GPU/mg., 64–1 = 3 GPU/mg. May, 1957—126–1 = <4 GPU/mg., 64–1 = 0.7 GPU/mg.

its potency at that time, its activity is manifestly less than 4 GPU/mg. Thus the activity ratio decreased from 20:1 to less than 5:1 after five months.

DISCUSSION

The data presented above indicate firstly, that refractoriness to the action of relaxin in guinea pigs develops only after repeated injections of the hormone, and secondly, that marked diminution in the sensitivity of guinea pigs to the action of porcine relaxin may be accompanied by a much smaller reduction in responsiveness to relaxin of pregnant rabbit serum. These facts suggest that the formation of an antirelaxin antibody is responsible for the loss in sensitivity which is observed. The fact that some decrease in sensitivity to 64-1 follows repeated injection of relaxin from sow ovaries indicates that the antihormone is of limited species specificity. In this connection, it is of interest to note that repeated administration of 64-1 to guinea pigs which have become refractory to porcine relaxin results in a rapid further loss in sensitivity to the former preparation.

Several interesting parallels may be drawn between the development of resistance to relaxin in guinea pigs and the appearance of antihormones in other species of animals. Thus, from Table 1 it appears that a significant decrease in responsiveness to relaxin can be detected within two to three months after beginning bi-weekly injections; a similar period of time was found by Maddock, *et al.* (4, 5) to be required for the appearance of antihormones to hog FSH in women.[4] The lack of absolute specificity of the anti-relaxin response is of interest in view of the fact that antigonadotropic sera appear to possess a rather low order of specificity generally, as judged by the ability of anti-FSH sera to inhibit heterologous pituitary and chorionic gonadotropins (4, 6, 7).

The observation that repeated injections of relaxin produce a refractory state in at least one species of animal, and that this phenomenon probably has an immunological basis assumes importance in view of the increasing clinical use of non-steroid ovarian preparations. Since the preparations used in this study were both chemically and biologically heterogeneous, the relative importance of relaxin and other protein components of the mixture for phenomena described above can be assessed only after further study.

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INACTIVATION OF ACTH BY ISOLATED RAT ADRENALS AND INHIBITION OF CORTICOID FORMATION BY ADRENOCORTICAL HORMONES[1]

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ABSTRACT

The response by isolated rat adrenals to ACTH is dependent upon the amount of ACTH added per unit weight of tissue and is independent of the concentration of ACTH in the medium.

Incubated rat adrenals respond to moderate doses of ACTH with an increased rate of corticoid formation for a limited time only.

Contact with adrenal tissue causes a decrease in amount and eventual disappearance of ACTH activity in the medium.

Exposure for five minutes to ACTH stimulates the corticoid formation by adrenals subsequently placed in ACTH-free medium.

These and other observations suggest that ACTH is bound and inactivated by adrenal tissue.

The formation of corticoids by rat adrenals is inhibited by the addition of medium in which other adrenals have been incubated, by the residue from a lipid soluble extract of such medium, and by crystalline compounds F and B. Concentrations of corticoid as low as $10^{-5}M$ are inhibitory, indicating the possibility that the inhibition of the pituitary-adrenal system by adrenal cortical steroids may occur, to some extent at least, at the level of the adrenal cortex.

FOR the study of factors influencing the corticoid production of the adrenal cortex the use of a system employing isolated adrenal tissue is of great advantage because the complications introduced by the presence of other tissues in the intact organism are avoided. Thus, by the use of the *in vitro* technique, it became possible to establish that calcium ions are required for the corticosteroidogenic response to ACTH (1), and that the response to ACTH is decreased or abolished by reversal of the sodium:potassium ratio, by anaerobiosis, and by inhibitors of the glycolytic pathway of glucose metabolism (2).

The studies reported here suggest 1) that ACTH is bound and inactivated by adrenal tissue and 2) that the inhibition of the pituitary-adrenal system by adrenocortical steroids may occur, to some extent at least, at the level of the adrenal cortex.

METHODS

Adrenals from 4 to 8 male Sprague-Dawley rats, weighing 200 gm. or less, were halved or quartered and incubated in Krebs-Ringer-bicarbonate-0.01 *M* glucose medium in the

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[1] Presented in part at the 1954 meeting of the Canadian Physiological Society.

Warburg or Dubnoff apparatus under 95% O_2–5% CO_2 at 38°. The adrenal tissue was distributed in such a way that each vessel contained a half or a quarter of an adrenal from each rat. After a preincubation period of 30 minutes to one hour the medium was discarded and the adrenals were reincubated with fresh medium, with or without additions of ACTH[2] and of substances whose effect on corticoid production was to be examined. Unless otherwise stated, the corticoid production during the following incubation period or periods was determined by measuring the ultraviolet absorption of a methylene chloride extract of the medium with a peak at 240 mμ (3), not just the absorption at 240 mμ.

The mixture of lipid soluble substances formed by the adrenal undoubtedly includes compounds that are not typically corticoids, as defined by Selye (4), and may include precursors or metabolites of corticosterone, which, according to Bush, is the principal corticosteroid of the rat (5). Previous papers from our laboratory showed that the lipid soluble substances formed by rat adrenals *in vitro* have the following properties.

1. They absorb ultraviolet light with a maximum at about 240 mμ (3, 6, 7).
2. More than half of the ultraviolet absorbing material reduces a substituted tetrazolium reagent (8).
3. Their production is increased by ACTH (2, 3, 6, 7, 8).
4. The increased production is dependent upon the amount of ACTH added (3, 6, 7).
5. They decrease the number of circulating eosinophiles in adrenalectomized mice (6).
6. After paper chromatography in two solvent systems the ultraviolet absorbing material with a polarity of that of corticosterone or greater represents more than 80% of the total ultraviolet absorption (8). The addition of ACTH results in an increased formation of all fractions but the greatest increase occurs in the area with the mobility of corticosterone.

We feel, therefore, that the total ultraviolet absorbing lipid is representative of the steroids produced by the adrenal cortex *in vitro* that are influenced by ACTH. For the survey of the many factors influencing the total adrenocortical response to ACTH the ultraviolet method is the most effective because of its simplicity, speed, sensitivity and precision. For a more detailed analysis of alterations in the formation of the various corticoids, paper chromatography (9), reduction of a substituted tetrazolium reagent, May and Baker 1767 (10)[3] and the Porter-Silber reaction (11)[4] were applied.

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[2] The doses of ACTH (Connaught, Lot 8-3) are based upon the label potency, 1.8 U./mg. However, an assay carried out after completion of this investigation indicated a potency of 1.4 U./mg. (3).

[3] In contrast to our earlier publications (8) we have followed Henley's procedure (10) closely. Aliquots of methylene chloride extracts containing 1.5 to 8 μg. of reducing lipid were taken to dryness and dissolved in 0.2 ml. of 95% ethanol at 38° for 30 minutes; 0.15 ml. of a reagent containing 1.5 mg. of 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (May and Baker 1767) per ml. of 95% ethanol was added, followed by 0.15 ml. of a reagent containing 0.3 ml. of 10% aqueous solution of tetramethyl-ammonium hydroxide in 10 ml. of 95% ethanol. After 20 minutes at 28° in the dark, 0.15 ml. of 0.045 M acetic acid in ethanol was added, and the absorption at 510 mμ was read in microcuvettes against a reagent blank and compared with two concentrations of standard compound F carried through the same procedure. The substituted tetrazolium chloride solution was prepared fresh daily.

[4] Aliquots of methylene chloride extracts, containing 2–6 μg. of Porter-Silber chromogens in 0.5 to 3 ml. of methylene chloride, were added to two tubes, tube A receiving 0.5 ml. of "blank reagent" (11), tube B receiving 0.5 ml. of "phenylhydrazine-sulfuric acid reagent with ethanol" (11). The tubes were shaken vigorously for 30 seconds, centri-

RESULTS

Effect of ACTH concentration

The response by rat adrenals to added ACTH is proportional, not to the concentration of ACTH in the medium, but to the amount of ACTH added per unit tissue. The following observations illustrate this point:

a) When the ACTH concentration in the medium is kept constant but the amount of ACTH per unit tissue is varied by varying the volume of medium, the corticoid production increases with the amount of ACTH per unit tissue (Fig. 1, lower part).

b) When the ACTH concentration is varied but the amount of ACTH per unit tissue is kept constant by varying the volume of medium inversely with the ACTH concentration, the corticoid production remains the same at all ACTH concentrations, or, if anything, is slightly lower at the higher ACTH concentrations (Fig. 1, upper part).

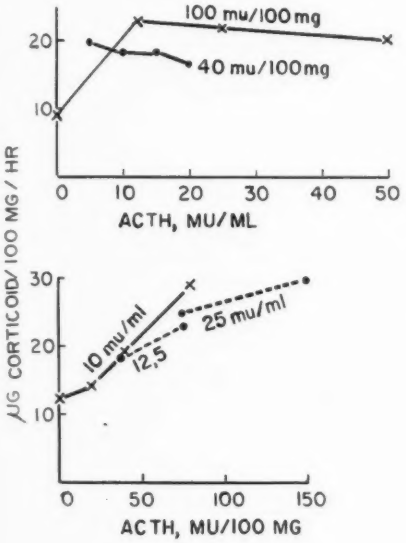

FIG. 1. Lack of correlation between ACTH-concentration of the medium and response to ACTH. The amount of tissue per flask was constant, about 30 mg. Upper part: Volume of medium varied inversely with the ACTH concentration, between 0.5 and 2 ml. Lower part: Volume of medium varied directly with the amount of ACTH added.

c) When the ACTH concentration and the volume of medium are kept constant, but the ACTH per unit tissue is varied by varying the amount of tissue, the corticoid production per unit tissue weight is higher in the

fuged, and the upper layer was aspirated and discarded. The tubes were stoppered and allowed to stand overnight in the dark. The next morning the color developed in tube A was read against the blank reagent, tube B against the phenylhydrazine sulfuric acid reagent, at 410 mμ. in microcuvettes, and the difference between tubes A and B was compared with the difference obtained with standard solutions of compound F carried through the same procedure.

TABLE 1. LACK OF CORRELATION BETWEEN ACTH-CONCENTRATION OF THE MEDIUM AND RESPONSE TO ACTH

a. Volume of medium constant at 1.5 ml.; ACTH concentration kept constant at 10 mU./ml.; tissue per flask varied

Mg. adrenals per flask		ACTH, mU./100 mg.		μg. corticoids/100 mg.	
A	B	A	B	A	B
23	66	53	18	27	19
22	46	54	26	17	17
32	88	62	23	37	21
36	102	55	20	31	23
26	50	60	30	23	19
30	62	51	24	33	21
		Mean diff. ±S.E.		8.0±2.3	
		P		<0.02	

b. Volume of medium constant at 1.5 ml.; ACTH per unit tissue weight kept constant at 25 mU./100 mg.; tissue per flask varied

Mg. adrenals per flask		ACTH mU./ml.		μg. corticoids/100 mg.	
A	B	A	B	A	B
15	43	2.5	7.5	42	35
14	48	2.5	7.5	24	20
8	33	1.3	5.4	17	19
14	28	2.3	4.7	17	19
16	42	2.7	7.0	17	19
18	45	2.6	7.5	25	23
		Mean diff. ±S.E.		1.2±1.4	
		P		>0.4	

Columns A refer to the flasks containing the lesser amount of tissue and Columns B, the greater.

vessels containing the smaller amount of tissue, i.e., the higher amount of ACTH per unit tissue weight (Table 1a).

d) When the ACTH concentration is varied in proportion to the tissue weight, keeping the volume of medium, and hence the amount of ACTH per unit tissue, the same, the corticoid production per unit tissue is also constant (Table 1b).

Time course of corticoid production

The corticoid production occurs at a fairly constant rate for about two hours[5] (Fig. 2). The rate then falls off and, with ACTH-stimulated adrenals (100 mU./100 mg. adrenals), reverts to the unstimulated rate. The corticoid production per unit adrenal weight is about the same whether halved or quartered adrenals are used.

Figure 3a shows the average time course, obtained in six experiments, of

[5] It should be emphasized that these time studies were carried out after a preincubation period of 30 minutes. During the preincubation as much corticoid appears in the medium as in the subsequent 90 minutes (12). During this period the adrenals are also much less responsive to added ACTH (7).

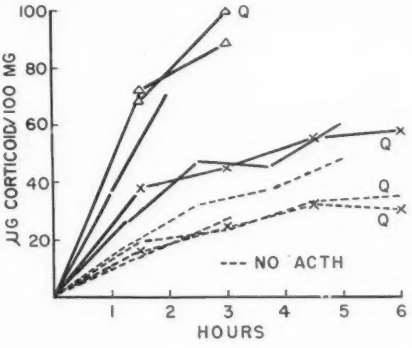

FIG. 2. Time course of the production of corticoids by rat adrenals *in vitro*. Similarly marked points indicate values obtained with tissue from the same animals. Curves marked Q were obtained with adrenal quarters, others with halved adrenals. ACTH concentration, 100 mu./100 mg.

corticoid production by quarters of the same adrenal tissue incubated without ACTH, and with 10, 30 and 90 mU. of ACTH per 100 mg. Four samples were incubated for 90 minutes, and four for 180 minutes. Figure 3b shows the average increase with ACTH during the periods of 0–90, 90–180, and 0–180 minutes.

During the first 90 minutes, all ACTH concentrations caused a significant increase in corticoid production, but there was no difference between the response obtained with 10 and with 30 mU. During the second 90 minute period, the corticoid production by adrenals incubated with the various doses of ACTH did not differ significantly from that of the unstimulated adrenals. At the lowest ACTH concentration the production during the second 90 minute period was actually below that of the unstimulated adrenals in most experiments.

Effect of adrenals on ACTH activity

The foregoing experiments showed that the effect of ACTH decreases

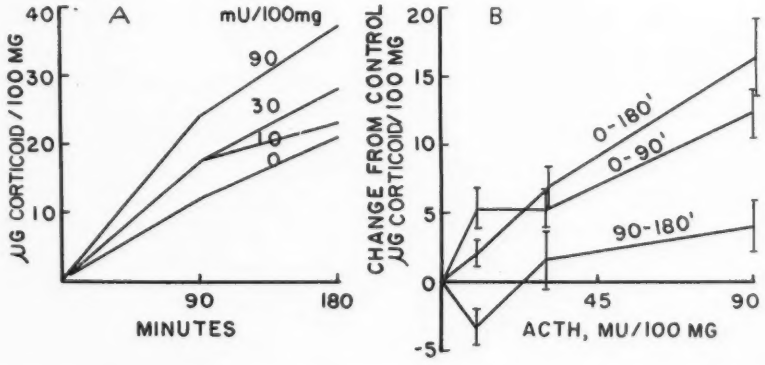

FIG. 3a. Time course of corticoid production with different doses of ACTH; 3b. Dose-response relationship at various periods of incubation. Bars indicate standard errors.

with time. The next experiments were designed to see whether ACTH-containing medium in contact with adrenals for one hour lost its ability to stimulate fresh adrenals.

Bisected adrenals were preincubated as usual and then incubated with 50 mu. of ACTH per 100 mg. adrenals. After one hour the medium was removed and assayed for ACTH activity on fresh quartered adrenals by the method of Saffran and Schally (3). Since preliminary experiments indicated that the activity was too low to ensure a reliable assay, known amounts of ACTH were added to the samples to be assayed. The results,

TABLE 2. LOSS OF ACTH ACTIVITY AFTER INCUBATION FOR ONE HOUR WITH RAT ADRENALS

ACTH previously incubated with tissue	Fresh ACTH added	Total ACTH expected	ACTH recovered by bioassay	95% confidence limits	Index of precision
		mu./100 mg.			λ
40	40	80	33	15–73	0.17
40	60	100	38	20–71	0.13
52	60	112	22	8–54	0.15
ACTH incubated without tissue					
35	—	35	37	18–77	0.16

summarized in Table 2, indicated not only a loss of ACTH activity, after one hour's incubation, but there appeared to be an inhibitory effect of the ACTH-containing medium, which had been in contact for one hour with adrenals, on the corticoid output of fresh adrenals, for not even the freshly added ACTH was recovered. On the other hand, there was no loss of activity when ACTH was incubated for one hour at 38° in Ringer medium without tissue.

Persistence of adrenal response after removal of ACTH-containing medium

That ACTH-containing medium in contact with adrenals for as little as five minutes apparently loses some of its activity is seen from the results of eight experiments, summarized in Table 3. Adrenals from four rats were bisected and distributed among four Warburg flasks in the usual way, each flask containing four adrenal halves contributed by four rats. After preincubation, one set of adrenal halves was incubated with ACTH-containing medium (100 mu./100 mg.) for 5 minutes at room temperature or at 38°. This medium was then added to the second set of adrenals, and replaced with ACTH-free medium. The third set of adrenals was incubated with freshly prepared ACTH, and the fourth set with ACTH-free medium. After one hour's incubation at 38°, the corticoid production in each of the four flasks was determined. The ACTH-containing medium that had been in contact with adrenal tissue for five minutes increased the corticoid production when added to another set of adrenal halves but was not as

TABLE 3. EFFECT OF 5 MINUTES' CONTACT WITH ACTH ON THE CORTICOID PRODUCTION BY RAT ADRENAL HALVES

	μg. corticoids produced during one hour		
In ACTH-free medium	With ACTH (100 mU./100 mg.)	After 5 min. contact with ACTH (100 mU./100 mg.)	In ACTH-containing medium previously incubated for 5 min. with other adrenal halves
a	b	c	d
14	28	18	28
6	44	20	31
13	31	17	25
12	19	15	13
15	28	23	30
13	34	14	26
9	30	17	24
10	35	22	18
Mean 11.5	31.1	18.2	24.4
Difference from control	19.6	6.7	12.9
		19.6	

Mean diff. ±S.E. between b and d	6.7±2.2	$p<0.02$
between a and c	6.7±1.6	$p<0.01$
between b and c	11.6±2.4	$p<0.01$

In the first three experiments the 5 minute contact with ACTH was carried out at room temperature; in all others at 38°. The incubations for one hour were carried out at 38°.

effective as freshly added ACTH medium. Adrenals in contact with ACTH for five minutes produced significantly more corticoids during the following hour than adrenals in ACTH-free medium but less than adrenals in contact with ACTH for one hour. It will be noted that the average increase in corticoid production by adrenals due to the short contact with ACTH plus the increase obtained with the ACTH-containing medium which had previously been in contact with adrenals for five minutes was equal to the increase obtained with fresh ACTH.

While adrenals in contact with 100 mU. of ACTH per 100 mg. for five minutes produce more corticoids when subsequently placed in ACTH-free medium than unstimulated adrenals, glands in contact for one hour with the same amount of ACTH, and then placed in ACTH-free medium, revert to nearly the unstimulated rate of corticoid production (Table 4). There was no consistent difference between the corticoid production by glands in contact with ACTH for one hour when they were thoroughly washed, or not washed at all before being placed in ACTH-free medium.

Inhibitory effect of ACTH-free adrenal incubation medium

It will be recalled that ACTH-containing medium in contact for one hour with adrenals not only had lost its activity but in some cases appeared to have an inhibitory effect when added to new adrenals (see Table 2). In the experiments summarized in Table 5, ACTH-free medium in contact

TABLE 4. EFFECT OF ONE HOUR'S CONTACT WITH ACTH ON THE CORTICOID PRODUCTION BY RAT ADRENALS

ACTH, 100 mu./100 mg.; μg. corticoids/hr./100 mg.

1st hr.	2nd hr.				1st hr.	2nd hr.
With ACTH	ACTH-containing medium replaced with ACTH-free medium				No ACTH	
	Glands not washed	Washed with 3.5 ml. medium				Medium replaced
		1x	3x	6x		
30	—	—	—	12	16	13
36	15	—	—	17	17	12
30	13	13	13	—	lost	8
29	19	19	12	—	16	12
Mean 31	16	16	13	14	16	14

with adrenals for 90 minutes was added to new adrenals and the corticoid production compared with that of adrenals from the same animals in fresh medium, all adrenals receiving either the same amount of ACTH, or no ACTH at all. In nine out of ten experiments the adrenals in medium in which other adrenals had been incubated produced less corticoids than adrenals in fresh medium.

Effect of periodic change of incubation medium

From these findings it was to be expected that a higher corticoid production would result if the medium were changed frequently. This was the case (Table 6). In the absence of ACTH more corticoids were produced when the incubation medium was changed than when it was not changed. When a given amount of ACTH was added at the beginning of the incubation period, less corticoids were produced than when the same amount was added in divided doses, removing the old medium and adding fresh medium each time. When ACTH was added in divided doses without changing the medium, again significantly less corticoids were produced than when the

TABLE 5. EFFECT OF ACTH-FREE MEDIUM IN CONTACT WITH ADRENALS FOR 90 MINUTES ON THE CORTICOID PRODUCTION BY FRESH ADRENALS

ACTH added, mu./100 mg.	μg. corticoids/100 mg./90 minutes	
	In fresh medium	In old incubation medium
—	17	6
—	13	11
25	25	19
50	49	30
50	34	26
50	50	34
75	41	13
100	31	28
100	27	30
100	36	28

TABLE 6. EFFECT OF CHANGE OF MEDIUM ON THE CORTICOID PRODUCTION BY RAT ADRENALS

			Corticoid production, μg./100 mg.				
	Incubation periods		Medium not changed		Med. changed each period	Diff. ±S.E.	P
	A. No ACTH						
(9)	2×45′		21.2		28.2	7.0±1.9	<0.01
	B. With ACTH						
		mu./100 mg.	Added at start	Added in divided doses at beginning of each period			
			Medium not changed		Medium changed each period		
(6)	2×45′	30	42.2	—	51.5	9.3±2.9	<0.05
(1)	6×20′	6	13	11	27		
(1)	4×30′	24	19	19	38		
(1)	2×60′	100	38	27	47		
(1)	2×60′	100	39	37	52		

Figures in parentheses represent number of experiments.

ACTH was added in divided doses but with a change of medium each time. There appeared to be little difference between the corticoid production when the ACTH was added all at once at the beginning of the incubation period, or in divided doses, without change of medium.

Effect of added corticoids

The possibility was considered that the corticoids liberated into the medium might have an inhibitory effect on further corticoid production. Rat adrenal incubation medium was therefore extracted with methylene chloride, taken to dryness, dissolved overnight in isotonic sodium chloride solution and added to fresh preincubated adrenals. In other experiments, crystalline compound F was added. After 45 minutes' incubation, the medium was removed and the adrenals were reincubated with a second dose of corticoids. The lipid extract from rat adrenal incubation medium, as well as crystalline compound F, inhibited the corticoid production, particularly during the second incubation period (Table 7).

Figure 4 shows the pattern obtained by scanning at 240 mμ papergrams of extracts of media from adrenals incubated with and without added compound F. 370 mg. of quartered adrenals were incubated in eight flasks, four flasks containing 12.3 μg. of compound F is one ml. medium, the others without added steroid. After one hour the medium was replaced with fresh medium. At the end of two hours the first and second hour incubation media were pooled, extracted with methylene chloride, and ali-

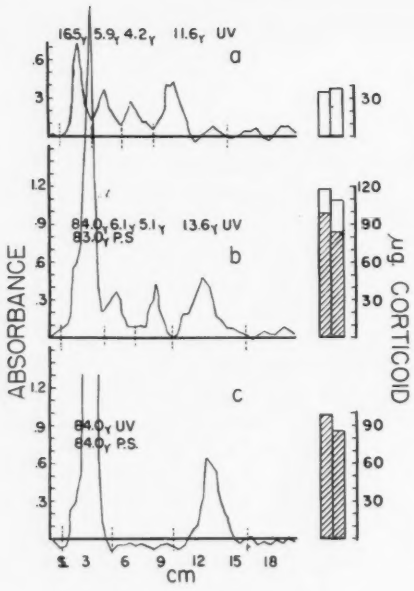

FIG. 4. Absorption at 240 mμ of paper chromatograms obtained from rat adrenal incubation media by the method of Burton *et al.* (9): a) Aliquot of medium in which adrenals had been incubated without addition of compound F. b) Aliquot of compound F-containing medium incubated with adrenals. c) Aliquot of compound F-containing medium not incubated with tissue and to which compound B had been added as marker after extraction with methyleen chloride. Values shown are calculated per total medium. Dotted lines along abscissae indicate position at which strips were cut. P.S. = Porter-Silber chromogens obtained from strips in the F region. UV = ultraviolet-absorbing lipid. Bars compare the total UV-absorbing lipid obtained before and after chromatography. Shaded area represents the amount of compound F added.

quots were chromatographed by the method of Burton *et al.* (9).

Four distinct peaks were observed, in agreement with the findings of Heard *et al.* (13) and of Eisenstein (14), both in the presence and absence of added compound F. The first and fourth peak corresponded to the position of authentic compounds F and B, respectively, on a control strip. The areas corresponding to the peaks were eluted separately with methanol and the ultraviolet absorption of the eluates was measured. In agreement with previous work from this laboratory (8), over 85 per cent of the total ultraviolet-absorbing material was recovered from the strips in areas of

TABLE 7. EFFECT OF ADDED CORTICOIDS ON THE CORTICOID PRODUCTION BY RAT ADRENAL GLANDS

μg. corticoids produced/100 mg. adrenal

		Control	+compound F.	Mean diff. ±S.E.	P
1st 45′	(6)	9.6	6.8	2.8±1.3	>0.05
2nd 45′	(6)	11.6	2.3	9.3±1.6	<0.01
		Control	+extracted corticoids		
1st 45′	(4)	9.2	5.5	3.7±0.9	<0.05
2nd 45′	(5)	11.4	2.4	9.0±1.8	<0.01

Figures in parentheses represent number of experiments. Compound F or corticoids extracted from rat adrenal incubation medium were added at the beginning of each incubation period. The amount added was 4–6 μg. per flask (volume of incubation medium, 1.5 ml.) or 20–30 μg. per 100 mg. adrenals.

mobility of that of compound B or less. The F region yielded 16.5 μg. of corticoid per 190 mg. tissue from medium to which no steroid had been added. However, from incubation medium to which compound F had been added, only the amount recovered from the control strip, 84 μg., was obtained, indicating complete suppression of the production of corticoid with mobility in the F region. The Porter-Silber chromogens in the eluate of the F region also equalled the amount of F recovered from the control strip. The areas corresponding to the remaining three peaks yielded values, by ultraviolet absorption, that were nearly the same in the presence and absence of added compound F.

Figure 5 is a typical comparison of the effect of added compounds F and B on the corticoid production. The methylene chloride extract of the medium after incubation was analyzed for α-β unsaturated ketones by the ultraviolet absorption method, for Porter-Silber chromogens, and for the ability to reduce a substituted tetrazolium reagent, May and Baker 1767.

The addition of compound F caused a complete suppression of the appearance of Porter-Silber chromogens, which could account for the decrease in both ultraviolet-absorbing materials and reducing substance. The addition of compound B suppressed the formation of α-β unsaturated ketones and reducing substances but had no significant effect on the Porter-Silber chromogens.

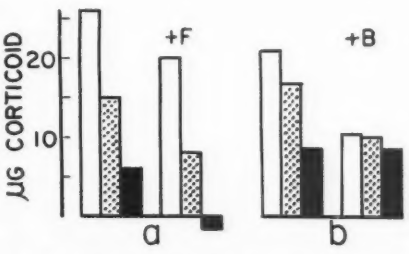

FIG. 5. Comparison of the effects of added compounds F and B on the production of UV-absorbing lipid (▭), lipids reducing M and B 1767 (▩) and Porter-Silber chromogens (■). a) 160 mg. tissue incubated for two hours with and without 15 μg. of compound F, changing the medium after the first hour. b) 130 mg. tissue incubated in the same manner with and without 25 μg. of compound B. Concentration of added steroid was 3.8 and 6.2 μg./ml. medium, tissue concentration about 40 and 32 mg./ml. medium, respectively.

DISCUSSION

Possible binding of ACTH

Observations in agreement with the concept that the binding of ACTH by the adrenal cortex and its inactivation occur during the process of stimulating corticoid formation are that 1) the response to ACTH is dependent upon the amount of ACTH per unit weight of tissue rather than upon the concentration in the medium; 2) the corticoid production by adrenals exposed to moderate doses of ACTH (less than 100 mU./100 mg.) is stimulated for a limited period only, and eventually reverts to the unstimulated rate, even though the adrenals are still fully capable of re-

sponding to ACTH; 3) short exposure (five minutes') of adrenals to ACTH stimulates the subsequent formation of corticoids in ACTH-free medium, and correspondingly decreases the ACTH-activity in the medium which had been in contact for the short period with the adrenals; 4) longer exposure (1 to $1\frac{1}{2}$ hours) results in complete loss of ACTH-activity in the medium at the dose levels used, and the corticoid formation by the adrenals subsequently placed in ACTH-free medium is no longer stimulated, indicating that the ACTH presumably bound or absorbed by the gland has become inactivated. However, with exposure to massive doses, two units per 100 mg. for one hour, Saffran and Bayliss (7) observed stimulated corticoid formation by adrenals subsequently placed in ACTH-free medium, analogous to our findings with short exposure at low doses.

Definite proof for the validity of this concept requires the demonstration of ACTH in adrenal tissue after exposure to the hormone. In the case of insulin, another polypeptide hormone, the binding by a target organ, muscle, has been proven unequivocally (15).

Inhibition by adrenocortical steroids

The suppression of the formation of corticoids by added compounds F and B and the evidence that the corticoids produced by the rat adrenal *in vitro* include these, or very similar, steroids, offer a good explanation for the observations 1) that the rate of formation of corticoids *in vitro* falls off with time, 2) that the rate is increased by a change in medium, 3) that medium from previously incubated adrenals inhibits corticoid production by fresh adrenals and 4) that the residue from a methylene chloride extract of medium from incubated rat adrenals is inhibitory. In each case, extra corticoids accumulate or are added.

The increase in corticoid production with change of medium explains the discrepancy of the results of our time course studies with those published by Saffran and Bayliss (7). The latter authors found a constant corticoid production over a 5-hour period as opposed to a decrease in production rate with time found in the present work. Saffran and Bayliss changed the medium at hourly intervals.

The inhibition by corticoids appears to be somewhat specific. Added compound F suppressed the formation of a compound F-like material; added compound B did not inhibit the formation of Porter-Silber chromogens, but affected the ultraviolet-absorbing and tetrazolium-reducing lipids. However, under certain conditions, and especially with higher concentrations, added compounds F and B completely suppress the formation of ultraviolet absorbing lipid (16) indicating the ability of these steroids to inhibit the formation of corticoids other than themselves.

The ability of adrenocortical steroids to suppress the adrenocortical response to stress is well known, but most workers feel that the site of action is in the pituitary or the hypothalamus (17, 18). However, the

sensitivity of the adrenal corticoid-forming system of the isolated rat adrenal gland to adrenocortical steroids is surprisingly high. The addition of 3–12 μg. of pure steroid per ml. corresponds to a concentration of 1.4×10^{-5} *M*. In the light of this sensitivity the possibility of an additional effect at the adrenal level of substances inhibiting the adrenal response to stress must be considered.

Identity of individual components

No attempt has been made in this study to characterize with certainty the individual components formed by the rat adrenal *in vitro*. Heard *et al.* (13) claim that the two major components are compounds F and B, while Eisenstein (14) presents evidence that the B spot consists of epicorticosterone. The amounts of Porter-Silber chromogen found in our work are surprisingly high in view of Bush's report (5) that the stimulated rat adrenal secretes mainly corticosterone. However, Eisenstein (14) found that 43% of the ultraviolet-absorbing material secreted by the rat adrenal *in vitro* gives the Porter-Silber test. This compares with 20–45% observed with unstimulated rat adrenals in our work. Hofmann (19) found that 26% of the formaldehydogenic material reacts with the Porter-Silber reagent, and has investigated the apparent inability of the rat adrenal to produce 17-hydroxylated corticoids in amounts comparable to other species.

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SERUM ELECTROLYTES IN THE HYPOPHYSECTOMIZED RHESUS MONKEY[1]

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ABSTRACT

Hypophysectomy in the rhesus monkey results in a small but statistically significant reduction in serum sodium concentration and a statistically significant rise in serum potassium concentration. Restriction in sodium intake, however, does not produce a further alteration in serum sodium and potassium concentration.

THE thesis that hypophysectomy in a variety of experimental animals and man, while leading to severe disturbances in carbohydrate metabolism, does not significantly alter serum sodium and potassium concentrations appears to be generally accepted. The ability of hypophysectomized animals to conserve sodium on a low sodium intake, however, is a matter of some controversy since one group of investigators (1) has found that this function in the hypophysectomized dog is essentially normal while another (2) also working with hypophysectomized dogs, reports a significant impairment in this regard.

The present report deals with the effects of pituitary ablation in the rhesus monkey on the concentration of sodium and potassium in the serum and the effects of a restriction in sodium intake on serum electrolyte levels.

METHODS

Twenty-five normal and twelve hypophysectomized rhesus monkeys of both sexes were utilized in this investigation. The animals were kept on Rockland monkey diet, supplemented with fresh fruits and vegetables, cooked rice and eggs. Water was provided *ad libitum* and in the case of hypophysectomized animals a 10% glucose solution was supplied in addition to tap water. Hypophysectomies were performed under pentobarbital anesthesia using the parapharyngeal approach. Completeness of the operation was determined at autopsy by examination of the sella turcica. The operated animals were given no replacement therapy. Serum sodium and potassium determinations were made with an internal standard flame photometer. Measurements were begun one month following hypophysectomy and continued, at intervals, for as long as 16 months. Serum electrolyte concentrations in intact, control animals were followed in like manner.

One rhesus monkey was maintained on a low sodium diet containing 0.01 meq. Na

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TABLE 1. EFFECT OF HYPOPHYSECTOMY ON SERUM SODIUM AND POTASSIUM CONCENTRATIONS IN RHESUS MONKEYS

	No. animals	No. observations	Serum sodium meq./L	P	Serum potassium meq./L	P
Normal	24	51	153.5 ± 0.5[1]			
Hypophysectomized	12	56	149.2 ± 0.7	< .01		
Normal	23	39			4.4 ± 0.1[1]	
Hypophysectomized	12	42			5.0 ± 0.1	< .01

[1] Mean ± standard error of mean.

per gm. (average daily intake of 1.5 meq) supplemented with the daily administration of 25 meq. of sodium by stomach tube in 20 ml. of water. During periods of sodium restriction the sodium intake was limited to the dietary source. Measurements of serum sodium and potassium concentrations were made during periods of high and low sodium intake before and after hypophysectomy.

RESULTS AND DISCUSSION

The data (Table 1) indicate that hypophysectomy in the monkey results in a small but statistically significant decrease in serum sodium concentration as well as a significant increase in potassium concentration. The magnitude of these changes was not correlated with the duration of the postoperative period. Restriction of the daily sodium intake to approximately 1.5 meq. per day was tolerated however, as well as before operation in terms of the stability of the serum sodium and potassium concentrations (Table 2). In contrast to the above, hypophysectomy in the rhesus monkey is followed by severe derangement in carbohydrate and protein metabolism (3, 4).

These data are in agreement with direct evidence which demonstrates that the adrenal cortical secretion of aldosterone in the rat and dog is in major part autonomous of adenohypophyseal function whereas the secretion of the glucocorticoids is markedly reduced in the absence of the pituitary gland (5, 6). Since the latter hormones contribute significantly to electrolyte homeostasis (6, 7) and since hypophysectomy results in some reduction in aldosterone secretion (5, 6), the observed shift in serum elec-

TABLE 2. EFFECT OF SODIUM RESTRICTION ON SERUM SODIUM AND POTASSIUM CONCENTRATIONS IN A MONKEY BEFORE AND AFTER HYPOPHYSECTOMY (SEE "METHODS" FOR DETAILS OF EXPERIMENT)

Treatment	Days	Observations	Serum sodium, meq./L	Serum potassium, meq./L
Normal				
High Na Intake	26	7	146.5 ± 1.4[1]	4.3 ± 0.1[1]
Low Na Intake	30	8	146.9 ± 1.3	4.4 ± 0.2
Hypophysectomized				
High Na Intake	28	6	143.0 ± 1.0	5.1 ± 0.1
Low Na Intake	30	9	142.1 ± 0.5	5.1 ± 0.2

[1] Mean ± standard error of mean.

trolyte concentrations in the direction of mild adrenal insufficiency following hypophysectomy in the rhesus monkey is not surprising.

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METABOLISM OF STEROIDS: THE REMOVAL OF EXOGENOUS 17-HYDROXYCORTICOSTERONE FROM THE PERIPHERAL CIRCULATION IN DOGS[1]

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ABSTRACT

A study was undertaken to determine whether the disappearance rate of exogenous cortisol could be used to evaluate the influence of experimental conditions or agents upon cortisol metabolism. It was found that the half-life of exogenous cortisol in a given dog was not very consistent from one day to another. Therefore, in a single experiment, the half-life of exogenous cortisol was obtained and then the influence of an administered agent upon this half-life was determined. The disappearance rate of exogenous cortisol was not influenced by subsequent administration of saline or dextrose. Neither a second injection of cortisol, nor small doses of ACTH (0.5–2.0 I.U.), altered the disappearance rate of cortisol, even though there was an increase in plasma concentration of the steroid. Larger doses of ACTH apparently caused a decrease in the rate of cortisol disappearance. Although this technic produces a "physiological hypophysectomy" it is not satisfactory for assay of ACTH. However, it does permit evaluation of the influence of conditions or agents on the disappearance rate of circulating cortisol.

IN STUDIES of adrenal function, with relation both to human disease and to animal experimentation, the direct measurement of circulating corticosteroids has been of great benefit. The use of this approach has revealed that abnormal 17-hydroxycorticosteroid (17-OHCS) concentrations exist in a variety of disease states (1, 2). However, the plasma steroid levels measured reflect not only the adrenal secretory rate but also the rapidity with which free steroid is removed from circulation by such factors as conjugation, degradation, excretion, and utilization. Consequently, for proper interpretation of data concerning circulating corticosteroid concentrations the factors of secretion and subsequent "metabolism" must be evaluated separately. Considerable information now exists pertaining to adrenocortical function, including steroid secretion, in different circumstances. In contrast, not much is known concerning the metabolism of the

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corticosteroids, once secreted. In studies reported to date, the disappearance rate of free steroid from the circulation has been found to be abnormal in a few disorders (3, 4). It seems probable that the rates of steroid metabolism may be abnormal in many disease states and the beneficial influence of some therapeutic agents may in part be mediated through their influence on these rates.

One approach to the study of steroid metabolism consists of administering exogenous steroid and determining the rate of its removal from circulation. When a pharmacological dose is administered, the removal of steroid from circulation corresponds to a first order reaction in that the rate is proportional to the concentration at any given moment. Therefore, the half-life of the steroid can be calculated and the rate of removal of steroid from circulation be expressed in terms of this half-life.

If, for a given animal, the half-life values obtained from day to day were sufficiently consistent, this technic could be used in animal studies to evaluate the influence of various experimental conditions, or of various drugs, on the rate of metabolism of administered steroid. Such an evaluation could be made by comparing the half-life value on a "control day" with that on an "experimental day." Therefore, the present study was undertaken to evaluate the consistency of the half-life values when 17-hydroxycorticosterone (cortisol) was administered to a given dog on each of many experimental days. Also, an experimental model was designed to allow evaluation of the influence of various agents on the half-life of administered cortisol by a single study performed on one day. The results and an evaluation of the practicality of this latter technic also will be presented here.

MATERIALS AND METHODS

Trained, unanesthetized mongrel dogs, weighing approximately 50 pounds, were used in these experiments. Since the dogs had a blood loss of approximately 200 ml per experiment, each animal was used not more than once per week and frequently was given longer rest periods to avoid anemia. Hematocrit values remained constant throughout each experiment. In these dogs free cortisol[3] was infused intravenously, 2 mg./kg. body weight. The steroid was prepared in 50% ethanol and diluted 15- to 20-fold with 5% dextrose in water. The infusion usually was completed in 12 minutes; however, for experimental reasons some studies were done with faster or slower rates of infusion. The moment the infusion was completed was recorded as zero time. Several successive heparinized blood samples were obtained from the external jugular vein at carefully recorded time intervals, varying between 10 and 30 minutes. The blood was centrifuged immediately after collection, and the plasma was separated and frozen until used. The 17-OHCS determinations were performed by the method of Nelson and Samuels (5) as modified by Eik-Nes *et al.* (6). In calculating the cortisol half-life for each experiment, the logarithms of the 17-OHCS concentrations were plotted against time and the straight line of best fit was determined by the method of least squares. From the equation for the

[3] Cortef Intravenous (R)—Generously supplied by Dr. C. J. O'Donovan, The Upjohn Company, Kalamazoo, Michigan.

regression line in each experiment, or in different parts of the same experiment, the half-life of cortisol was determined. In order to minimize the influence of physiological variations in plasma 17-OHCS concentrations, no plasma value smaller than 10 μg.% was used in the computation of the regression lines.

In one series of experiments the influence of injected agents on the cortisol half-life was evaluated. The agents used were saline (1–25 ml.), 5% dextrose in water (1–25 ml.), cortisol (1–10 mg.), and ACTH (0.1–10.0 I.U.).[4] In these experiments 5 or more samples of blood were obtained following the initial infusion of cortisol, so that the baseline half-life value could be calculated. Immediately after the last of these blood samples was drawn, the test material was injected intravenously and 5 to 7 more blood samples were obtained. In this way the cortisol half-life before and that after the second injection could be compared under essentially the same experimental conditions.

RESULTS

Blood samples were taken before the cortisol infusion in many experiments to determine the concentrations of endogenous 17-OHCS. These data are shown in Table 1. In 47 samples obtained from 6 dogs the mean concentration was 1.7 ±0.14 μg./100 ml. plasma.[5] There were no significant

TABLE 1. PLASMA 17-OHCS CONCENTRATIONS IN DOGS

Dog	No. determinations	Plasma 17-OHCS (μg./100 ml.)		
		Mean	S.E.M.	Range
1	11	2.0	±0.92	0.0– 9.8
2	9	1.9	±0.65	0.0– 4.6
3	10	2.5	±1.05	0.0–10.2
4	7	1.2	±0.70	0.0– 4.8
5	6	0.9	±0.34	0.0– 1.9
6	4	1.0	±0.91	0.0– 3.7
	47	1.7	0.14	0.0–10.2

differences among the mean values observed in the individual dogs. With the exception of the first sample obtained from each of two dogs (in which the plasma 17-OHCS concentrations were 9.8 and 10.2 μg./100 ml. respectively) all plasma samples contained concentrations of 17-OHCS less than 6.0 μg./100 ml. It seems that the two high values might be attributable to apprehension. In 18 of the 47 samples no 17-OHCS could be detected, although satisfactory volumes of plasma were used (an average of 10.1 ml.). Although in human subjects a marked diurnal rhythm in 17-OHCS concentration has been demonstrated (8–10), this was not found in these dogs; however, the 17-OHCS values are so low that any diurnal variations present likely would not be detected.

The results of 80 half-life determinations of exogenous cortisol in 4 dogs are shown in Table 2. Here, all of the individual half-life values for each dog are presented. As can be seen, the range of half-life values in a given

[4] International Units.

[5] Eik-Nes and Brizzee (7) have reported a mean value of 5 ±0.37 μg.% in anesthetized dogs.

TABLE 2. SERIAL HALF-LIFE VALUES FOR EXOGENOUS CORTISOL IN INDIVIDUAL DOGS*

Cortisol half-life (minutes)						
Dog. No. 1		Dog. No. 2		Dog. No. 3		Dog No. 4
72	55	49	55	59	57	61
48	35	30	45	51	52	76
45	42	53	43	81	52	67
44	42	50	48	47	56	50
62	69	56	70	61	65	60
57	42	31	54	51	44	67
59	54	40	58	67	51	
28	59	40	53	57	46	
50	56	39	52	55	56	
49	48	47	40	45	60	
70	58	45	42	47	64	
43	38	43	48	48		
57		42	88			

* These serial half-life studies for a given animal were performed at intervals of one week or longer in every instance.

animal was considerable even though the conditions of the experiment were kept as constant as possible. The magnitude of this range reflects the occasional widely divergent values obtained in each dog; however, the majority of values for each animal fell within ± 10 minutes of the mean for that animal and consequently the standard error of the mean for each animal was quite small (Table 3). The mean cortisol half-life value for all dogs was 52 ± 1.2 minutes.[6] Although the mean values for individual dogs did not differ much from this, they did differ significantly among themselves. Evaluation of these data by analysis of variance indicates that the probability that the differences of the mean values among these dogs are due to chance alone is less than 2%.

The data in Figure 1 indicate that the variation in the individual half-life values for a given dog was not dependent upon the rate of cortisol

TABLE 3. HALF-LIFE OF EXOGENOUS CORTISOL IN DOGS

Dog	No. determinations	Half-life (minutes)		
		Mean*	S.E.M.	Range
1	25	51	±2.2	28–72
2	26	49	±2.3	30–88
3	23	55	±1.8	44–81
4	6	64	±3.6	50–76
All dogs	80	52	±1.2	28–88

* Analysis of variance indicates probability that differences among dogs are due to chance alone is less than 2% but greater than 1%.

[6] This value is only approximately 50% as great as that in man (3, 11), indicating that cortisol is metabolised much more rapidly by the dog than by man. It is not known whether this difference pertains equally to all steps involved in cortisol metabolism or whether it is attributable chiefly to some certain step.

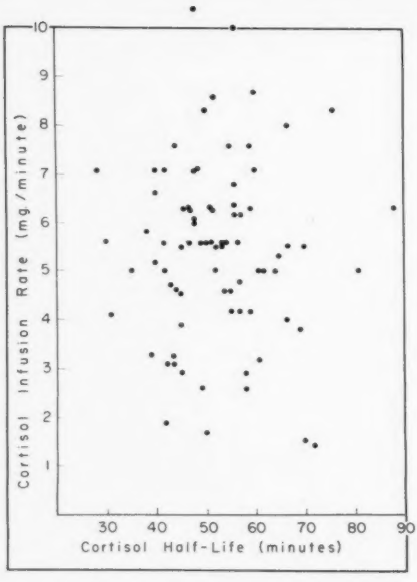

FIG. 1. Relation of cortisol infusion rate to cortisol half-life value obtained.

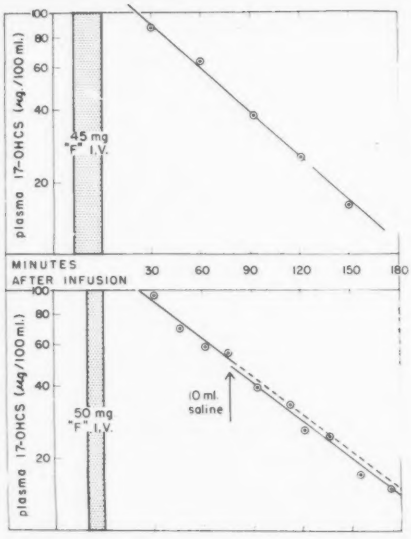

FIG. 2. Disappearance rate of exogenous cortisol, and influence of saline injection on this footnote: "F" = cortisol.

infusion. This figure represents a scattergram relating the individual cortisol half-life values in the 4 dogs to the rate of cortisol infusion, with the latter being varied from 1.4 to 10.4 mg./minute. These data fail to demonstrate any correlation between the two variables.

Figure 2 shows graphically the data from two typical experiments illustrating the rate of disappearance of exogenous cortisol and the effect of a saline injection upon this rate. In the upper portion of the figure are shown the data from a standard experiment for determining the half-life of cortisol. In this experiment 45 mg. cortisol was infused intravenously and blood samples were obtained at 30, 60, 90, 120 and 150 minutes after the infusion was completed. The logarithms of the 17-OHCS concentrations were plotted against time. In the lower portion of the figure are shown data illustrating the effect of a saline injection on the rate of disappearance of cortisol from the circulation. After 50 mg. cortisol was infused intravenously, enough blood samples were obtained to determine the slope of the disappearance curve; then saline was injected intravenously and several more blood samples were obtained. As can be seen, the slope of the regression line was not affected appreciably by the injection of saline.

Table 4 summarizes the data obtained when experiments of this type were performed to determine the effect of various amounts of saline or of 5% dextrose on the half-life of cortisol. In addition, data are shown for 7 control experiments in which plasma samples were obtained at correspond-

TABLE 4. INFLUENCE OF SALINE OR DEXTROSE ON THE HALF-LIFE OF EXOGENOUS CORTISOL

Substance injected	Number of experiments	Mean half-life cortisol		
		Before injection	After injection	Difference
Saline Solution	8	55.4	59.0	+3.6±3.0
5% Dextrose Solution	5	49.4	54.4	+5.0±3.1
No Injection	7	58.9*	61.4†	+2.5±4.2

* 1st portion of curve.
† 2nd portion of curve.
(In each portion the regression line represents a minimum of 5 points.)

ing time intervals following cortisol infusion but in which no second injection was given. Enough samples were obtained after the infusion of cortisol so that the regression line could be divided into two portions and cortisol half-life determined independently for each portion. As shown in Table 4, the differences in half-life between the first and second portions of the curve were of the same order of magnitude whether or not an injection had been given. Thus it seems apparent that neither saline nor 5% dextrose in the amounts employed influence the rate of disappearance of exogenous cortisol from the circulation.

Figure 3 illustrates the effects of different amounts of cortisol on the disappearance curve. The data shown were taken from representative experiments. It will be noted that at either dose level shown little change occurred in the slope of the regression line following the second injection of cortisol; thus the half-life of cortisol remained constant. However, the curve was shifted to the right and the magnitude of this shift was dependent upon the amount of cortisol injected. In a very short time after the second injection, equilibrium was reached and the disappearance of cortisol at its preceding rate was resumed.

The effects on the cortisol disappearance curve produced by various amounts of ACTH are illustrated in Figure 4 and in Table 5. As shown in

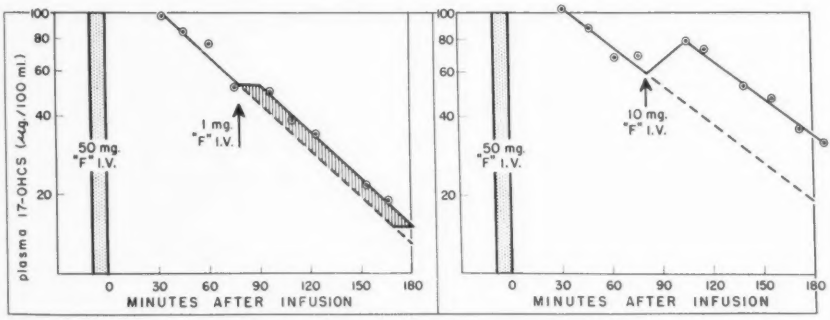

FIG. 3. Influence of a second cortisol injection on the cortisol disappearance rate.

TABLE 5. THE INFLUENCE OF INJECTED CORTISOL OR ACTH ON THE HALF-LIFE OF EXOGENOUS CORTISOL

Group	No. experiments	Half-life (minutes)		
		Before	After	Difference
Control	20	55.1	58.7	3.6±2.0
Cortisol (1–10 mg.)	11	49.4	52.8	3.5±3.5
ACTH (I.U.)				
0.1	2	45	47	2
0.25	3	52	57	5
0.5	4	48	52	4
1.0	4	47	50	3
2.0	4	44	50	6
5.0	4	42	74	32
10.0	3	63	148	85

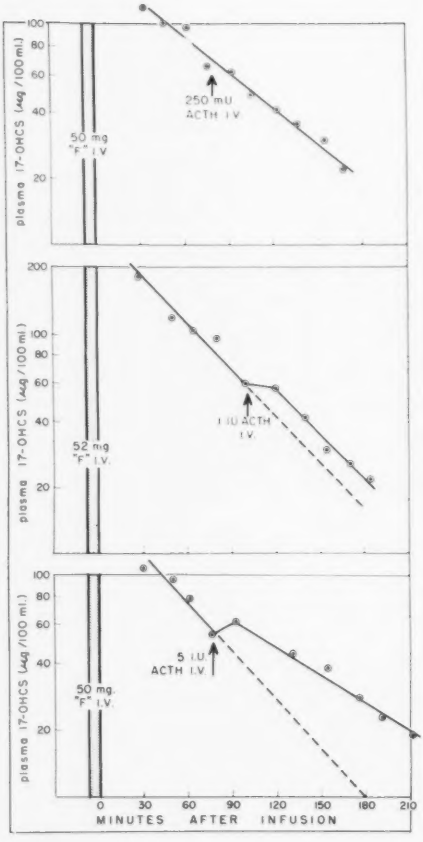

FIG. 4. Influence of various amounts of ACTH on the cortisol disappearance rate.

Figure 4, with 250 milliunits of ACTH, no measurable effect was produced. With one I.U. of ACTH no appreciable change in the slope of the regression line was demonstrated by the regression line did shift to the right, as it did with a second cortisol injection. Again, equilibrium apparently was reached in a very short time and the disappearance of cortisol continued at its preceding rate. However, when a larger amount of ACTH (5 I.U.) was injected, not only did the disappearance curve shift to the right but an obvious change in its slope appeared, resulting in unequivocal prolongation of half-life.

Thus, in response to a second injection the disappearance curve may be shifted without affecting its slope (half-life unaltered) or it may be shifted and its sloped changed (half-life altered). In Table 5 the cortisol half-life before and that after the injection of a second agent are compared. Saline, 5% dextrose, cortisol or ACTH was used for the second injection. In "control" experiments (saline or dextrose injec-

TABLE 6. THE INFLUENCE OF INJECTED AGENTS UPON THE AREA* BETWEEN THE REGRESSION LINES REPRESENTING THE CHARACTERISTICS OF EXOGENOUS CORTISOL REMOVAL

	Saline	5% dextrose	Cortisol		ACTH	
No. experiments	8	5	11		22	
	Surface area-cm.²		Dose (mg.)	S.A.-cm.²	Dose (I.U.)	S.A.-cm.²
	2.0	3.0	1	8.6	0.1	4.3
			2	11.4	0.25	3.2
			5	15.7	0.5	7.6
			10	22.4	1.0	8.6
					2.0	11.5
					5.0	27.0
					10.0	32.5

* These areas measured as indicated in the text (cf. footnote 7).

tion or no injection), the second half-life was essentially the same as the first. Also, when cortisol was given as the second injection, regardless of dose, there was a change only in plasma concentration but not in half-life. With ACTH, in amounts up to 2 I.U., the cortisol half-life again was not changed. With doses of 5 and 10 I.U., however, considerable prolongation of half-life occurred.

Even though the cortisol half-life may be unaltered following the injection of a second agent, any shift in the disappearance curve can be evaluated. In order to quantitate this effect in comparing various agents the data were plotted on standard graph paper[7] and the surface area between the two regression lines (shaded part in curve showing effect of 1 mg. cortisol in Figure 3) was measured. To standardize these surface area evaluations 17-OHCS concentrations of 50 and 15 μg.% were used as superior and inferior limits. Thus the area measured represents a trapezoid bounded superiorly and inferiorly by these 17-OHCS concentrations and laterally by the two regression lines. This surface area was expressed as cm². Table 6 shows the influence on this area produced by injection of various agents. With a second injection of saline or 5% dextrose this surface area is negligible. With a second injection of cortisol the area increases with increasing doses. With doses of ACTH less than 0.5 I.U. the area does not differ from that in control experiments. Above 0.5 I.U. the area increases with increasing dose.

DISCUSSION

Adrenal corticosteroid secretion has been studied experimentally by perfusion technics and by catheterizing the adrenal vein *in vivo* (12). The investigation of steroid metabolism by measuring the disappearance rate of administered steroid allows additional evaluation concerning the fate of steroids after their secretion and promises to be a valuable adjunct in the

[7] No. 340–L210 Dietzgen semi-logarithmic graph paper (2 cycles×10 divisions per inch) was used. On the abscissa the scale used was one inch = 30 minutes.

overall study of steroid relationships in health and disease. In certain disease states studies of this type have been performed. Peterson *et al.* (3) have shown the cortisol half-life to be altered in thyroid disturbances; Brown *et al.* (4) have reported this also in liver disease and Done *et al.* (13) in active rheumatic fever. However, except for the reports by Tyler *et al.* (14) and Hughes and Ely (15) on the effect of age, there has been little investigation of factors other than disease which may influence these disappearance rates.

For investigating the influence of factors other than disease it would be desirable to compare the half-life of exogenous cortisol under certain experimental conditions with the half-life on a control day. To permit this type of comparison, consistent half-life values under control conditions must be obtainable from one day to another in a single animal, even though differences may occur among different animals. In the 4 dogs studied here the differences among the mean cortisol half-life values for the different animals probably reflect actual differences in the individual rate of metabolism of this steroid. In each animal the half-life values were fairly reproducible from day to day. Occasionally, however, the half-life on a given day differed considerably from those found generally in that animal. This divergence was not caused by variations in the rate of cortisol infusion; neither was it related to the activity of the dog. Errors in timing of sample collections, or in the method itself, might account for the disparity; however, the constancy of the regression line slope, a product of serial plasma sample determinations, mitigates against such an interpretation. From these results it seems clear that unless more consistent control half-life values can be obtained for a single animal, comparison of the half-life value obtained on a control day with that obtained on another day when some substance is injected or the animal is subjected to stressful circumstances cannot be made reliably.

On the other hand, it appears entirely practical to study the influence of an injected agent, and possibly of a stressful insult, on the disappearance rate of circulating steroid, providing these factors are fused into a single day's experiment. Each animal then serves as his own control, and the factors which cause day-to-day variability are eliminated. In such an experimental design, several plasma samples are obtained following steroid administration and the control half-life value is determined from the slope of the regression line. Any change in this slope subsequent to injecting other agents or stressing the animal, can be attributed to the experimental procedure and evaluated quite reliably. In this study (Table 4) the cortisol half-life values, as calculated from the slopes of different portions of the regression line in a single experiment, agreed very well. The injection of saline or dextrose did not influence these values appreciably. These observations provide the basis for a practical technic for studying the effect of

various drugs and hormones, and possibly stressful factors, on the production and metabolism of cortisol and other steroids.

It was postulated originally that in this type of experiment a second injection of cortisol would alter the concentration but not the disappearance rate of this steroid. As mentioned, the data obtained were in accord with this postulation. It was postulated further that, if the adrenal cortex is capable of responding to exogenous ACTH in the presence of elevated circulating cortisol levels, the effect of an injection of ACTH on the cortisol disappearance curve should be similar to that following a second cortisol injection. If this latter were the case, it would form the basis for a potential ACTH assay technic measuring steroidogenesis directly. Such a technic would have several obvious advantages over present ACTH assay methods, the most satisfactory of which are based on the ability of ACTH to cause adrenal ascorbic acid depletion in hypophysectomized rats (16). This depletion is assumed to have a direct relation to adrenal function in terms of adrenal corticosteroid production. However, that a *direct* relationship exists is problematical (17–20). Furthermore, surgical hypophysectomy is a mutilating operation which often results in incomplete removal of the gland. Even though the greatest care is used in performing the operation it always is necessary to autopsy every animal to be sure that no fragments of pituitary tissue remain. If such pituitary "tags" do remain, they critically affect the responsiveness of the adrenal gland during the post-hypophysectomy period (20). A method of ACTH assay which could be performed without surgical hypophysectomy not only would avoid any disadvantages of the latter but also would result in a less complex procedure.

It was hoped that the method reported here using a "physiological hypophysectomy" with exogenous cortisol would provide a satisfactory assay for ACTH based upon its steroidogenic potency. However, this did not prove to be true. Not only is considerable variation evident in the effect of the same ACTH dose in different animals, but the range over which a *simple* dose response relationship exists is only from 0.5–2.0 I.U. ACTH. With larger amounts of ACTH the slope of the disappearance curve becomes altered so that the surface area between the regression lines increases disproportionately rapidly. This change in slope of the regression line after large amounts of ACTH have been injected probably indicates that the release of steroids by the adrenal cortex continues for an appreciable time after it has been so stimulated. It also is possible that this change in slope could in part reflect a direct effect of ACTH in decreasing the rate of disappearance of cortisol from the circulation, but there is no evidence available with regard to this possibility. However, the chief disadvantage of the method lies in its lack of sensitivity. The influence of ACTH in doses smaller than 0.5 I.U. is not detectable. This sensitivity compares unfavor-

ably with that of adrenal ascorbic acid depletion methods, which can assess as little as 0.00025 I.U. ACTH (16). Likewise, it compares unfavorably with the sensitivity of the method measuring steroidogenic potency in hypophysectomized dogs proposed by Nelson and Hume (21). Although this latter method is technically difficult to perform, it is without doubt of greater practical value than the method presented and evaluated in the present communication.

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INFLUENCE OF CORTISONE ON THE KETOGENIC RESPONSE TO OXYCEL CORTICOTROPIN[1]

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ABSTRACT

Cortisone inhibits fasting and certain other types of ketosis while oxycel corticotropin stimulates ketosis in both the fasted and fed rat, presumably by an extra-adrenal mechanism. Pretreatment of rats with 5 mg. of cortisone acetate daily for two or five days prevented the ketosis observed with a subsequent 24-hour fast as well as that following 3½ hours of Nembutal anesthesia, but did not modify the ketosis consequent to an injection of oxycel corticotropin. Since the cortisone treated animals presumably had ample liver glycogen stores it is concluded that the ketogenic action of corticotropin must be fundamental to either ketone production or utilization rather than secondary to some primary alteration in carbohydrate metabolism.

PRETREATMENT of normal rats with cortisone or hydrocortisone prevents the development of ketosis during fasting (1), cold exposure (2) or poisoning with sodium fluoracetate which blocks the Krebs cycle (3). On the other hand, these steroids are ineffectual in preventing the ketosis which follows insulin hypoglycemia in the rat (4) and in man (5). The doses of corticoids used did not prevent the marked depletion of liver glycogen which accompanies hypoglycemia in the rat and hence a reasonable basis for the subsequent development of ketosis persisted.

Oxycel corticotropin has been found to be a potent stimulus to ketosis. This reaction is not mediated by the adrenal cortex being demonstrable in the adrenalectomized rat (6). The ketogenic action of oxycel corticotropin can be shown to persist even in the presence of a glucose load since the hormone prevents the usual suppression of fasting ketonemia when glucose is infused intravenously. Although the corticotropin lowers the blood sugar and improves glucose tolerance it does not depress liver glycogen levels. Depletion of carbohydrate stores in the liver is therefore not a contributing factor in this ketosis as it appears to be in insulin hypoglycemia ketosis (4, 7).

The present study was designed to determine whether cortisone, which

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suppresses fasting ketosis and elevates the liver glycogen, would modify the ketosis consequent to corticotropin administration.

METHODS

Male albino rats weighing 200–250 gm, and fasted 18–24 hours were used. Corticotropin was injected intraperitoneally into rats kept under light Nembutal anesthesia at a constant temperature of 33° C. Blood ketone levels were estimated in tail blood by the technique of Werk *et al.* (8) before and $3\frac{1}{2}$ hours after corticotropin injection.

RESULTS

Figure 1 illustrates the ketogenic response to 0.3 mg. of Wilson's oxycel corticotropin (Lot 90834) in untreated rats and in animals that had received 5 mg. of cortisone acetate subcutaneously for 2 and 7 days respectively. The clear bars represent the increase in blood ketones in $3\frac{1}{2}$ hours in rats which did not receive ACTH while the response to ACTH in each case is charted to their right. The data in the untreated group confirm previous results, i.e. a significant increase in blood ketone levels occurs during a $3\frac{1}{2}$ hour period of Nembutal anesthesia and is substantially accentuated by corticotropin. In unanesthetized rats there is no increment in blood ketones during a comparable period of observation and a considerably larger dose of ACTH is necessary to initiate a ketonemic response (6, 9). Treatment with cortisone significantly depressed the ketonemic response to anesthesia but did not diminish the increment in ketonemia consequent to corticotropin

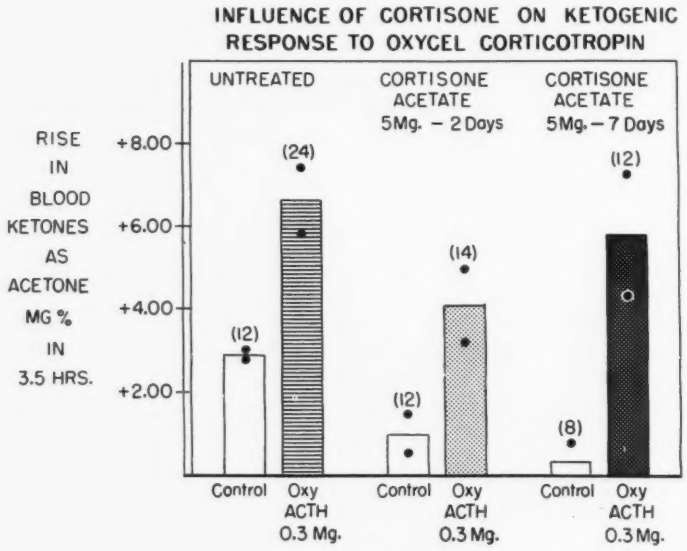

FIG. 1. Influence of intraperitoneal oxycel ACTH injection on blood ketone levels in fasted rats, untreated and after 2 and 7 days of cortisone acetate treatment. The rats were kept under light Nembutal anesthesia.

injection, even though the final blood ketone levels were somewhat less in the cortisone treated rats.

DISCUSSION

This study presents one situation in which ketosis is suppressed by cortisone treatment and another in which it is not. The mechanism of the ketosis associated with Nembutal anesthesia and its blockade by cortisone are as yet unexplained. Another barbiturate, phenobarbital, has been found to have a similar action (10). In all likelihood the ketosis is a reflection of an action of the barbiturates on acetate and lipid metabolism in the liver (11, 12).

In contrast to its influence on the ketosis associated with barbiturate anesthesia, cortisone was entirely without effect on the ketosis secondary to the action of oxycel corticotropin. Under the conditions of this experiment it may be assumed with confidence that the liver glycogen levels of the cortisone treated animals must have been quite ample. Hence, as in the previous study in which the antiketogenic effect of glucose was prevented by oxycel corticotropin (7), it would appear that the action of oxycel corticotropin must be quite fundamental to either ketone production or utilization rather than representing a reflection of some primary change in carbohydrate metabolism. At the present time the data are inadequate to warrant further speculation as to the mode of action of the oxycel corticotropin. However, it should be emphasized again that this action is not mediated by the adrenal cortex and that it may represent a special property of oxycel corticotropin and certain corticotropin peptides and not necessarily the naturally occurring hormone (13, 14).

Acknowledgment

We are indebted to Dr. David Klein, the Wilson Laboratories, Chicago, Illinois for the oxycel corticotropin.

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URINARY GLUCOSE AND TUMOR GROWTH IN PARTIALLY DEPANCREATIZED FORCE-FED RATS

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ABSTRACT

This was a study of urinary glucose and tumor (Walker carcinoma 256) growth in partially depancreatized rats. The rats were force-fed in most of the experiments. The experiments involved 204 partially depancreatized and 60 normal rats. The rate of tumor growth is normal in mildly diabetic rats, but when glycosuria is moderate or severe, tumor growth is retarded in proportion to the severity of the diabetes. As the tumor grows, the glycosuria becomes less severe in all the diabetic tumor hosts. When severely diabetic rats eat *ad libitum*, the rate of tumor growth is suppressed; the glycosuria is markedly decreased as the tumor grows, and the voluntary intake of food, initially very high, is decreased to subnormal levels. When the diabetes of the depancreatized rat is controlled by insulin, the tumor grows at a normal rate. An attempt was made to cause death of the tumor by producing severe diabetes. Although the Walker tumor degenerates more extensively in the diabetic rat than in nondiabetic rats, it was not found possible to destroy the tumor completely by this means, or to prolong the survival of the tumor host significantly by beginning the administration of small doses of insulin two weeks following implantation of the tumor. When the administration of insulin is started, the tumor begins to grow rapidly.

IN A STUDY of the relationship of alloxan-diabetes and tumor growth in the rat, Goranson and Tilser (1) found a reduction in the incidence and size of transplanted tumors. The rate of tumor growth bore an inverse relationship to the severity of the diabetes. When the tumors were transplanted intraperitoneally before or after the administration of alloxan, the diabetic action was either mitigated or there was amelioration of the diabetes. These authors have reviewed the pertinent literature.

More recently Ingle (2) reported that the growth of the Walker Carcinoma 256 is retarded in proportion to the severity of the glycosuria in force-fed partially depancreatized rats and that as the tumor grows the glycosuria becomes less severe. The present study confirms and extends these observations.

METHOD

Male rats of the Sprague-Dawley strain were subjected to partial pancreatectomy at a weight of approximately 375 gm. The procedure of Ingle and Griffith (3) was modified in order to remove the pancreas from within the duodenal loop by gentle aspiration with

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a fine pipet. Any amount of pancreas can be removed within 10 to 15 minutes. When the pancreatectomy is complete or nearly complete, the animal must be treated with insulin in order to survive. Varying amounts of pancreas were removed in these experiments. Insulin was not used postoperatively and some of the most severely diabetic rats died. During the first week following operation all of the rats were given physiological saline to drink and were given 5000 units of penicillin and 5 mg. of streptomycin per rat per day.

Following a postoperative period of at least 6 weeks, the animals were placed in metabolism cages and adapted to the force-feeding of a medium carbohydrate diet (4) by stomach tube each morning and late afternoon (26 cc. per rat per day). Twenty-four-hour samples of urine were collected at the same hour each morning and were preserved with thymol. Urine glucose was determined by the method of Benedict (5). Following a control period, an undiluted homogenate of Walker carcinoma 256 was injected into the upper thigh of each hind leg.

EXPERIMENTS AND RESULTS

Experiment 1 (Fig. 1) involved 120 diabetic rats and 12 normal rats. The diabetic rats were divided into 5 groups of 24 rats each according to amounts of glucose excreted during the control period. Twelve rats from each group then received tumor implants and the remaining 12 rats in each group continued to serve as controls. Twelve normal rats also received implants of tumors. The rats were killed 12 days following the implantation of tumors. The tumors grew at a normal rate in depancreatized rats which had either a mild glycosuria or no glycosuria. In rats which excreted 2.5 gms. or more of glucose daily, the growth of the tumor was suppressed to an extent proportional to the severity of the glycosuria. There was a striking suppression of glycosuria in the tumor hosts, whereas the glyco-

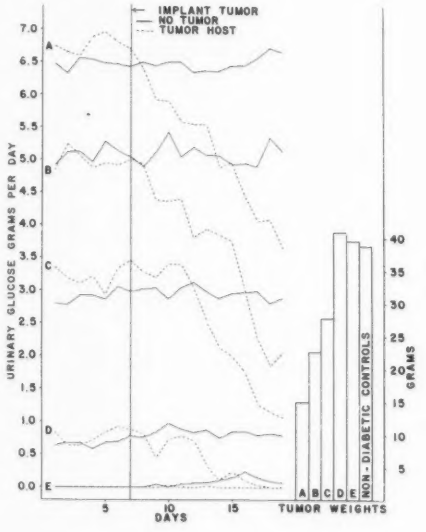

FIG. 1. Relationship between urinary glucose and tumor weight in force-fed rats. Averages for 12 rats per group.

suria of the controls in each group remained uniform.

Experiment 2 (Fig. 2) involved 24 normal rats and 24 moderately diabetic rats. The rats were tube-fed during the control period and were then permitted to eat the fluid diet *ad libitum* for 12 days. At the beginning of eating *ad libitum*, half the rats in each group received implants of tumor. There was a striking increase in food intake by the diabetic rats and a small increase in food intake by the normal rats when they were permitted to eat *ad libitum*. There was an accompanying increase in glycosuria which was subsequently suppressed in the tumor hosts. The diabetic and non-diabetic tumor hosts showed decreased appetite as the tumor grew. The tumors grew to a much larger size in the non-diabetic than in the diabetic rats.

Experiment 3 (Fig. 3) involved 12 non-diabetic and 12 rats having diabetes of moderate severity which were each implanted with tumors and observed until death. Here again the tumors grew to a greater size in the non-diabetic animals and the glycosuria of the depancreatized tumor hosts decreased with time. One exception was noted in which the glycosuria first decreased and was then exacerbated until the rat died in diabetic coma. The tumor was found to be very small and was apparently completely

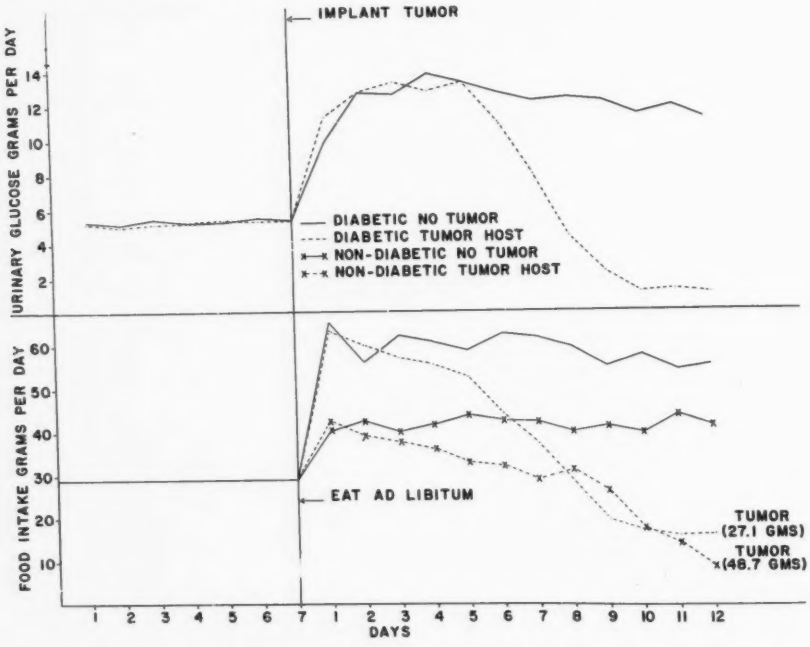

FIG. 2. Relationship between urinary glucose and tumor weight in rats eating *ad libitum*. Averages for 12 rats per group.

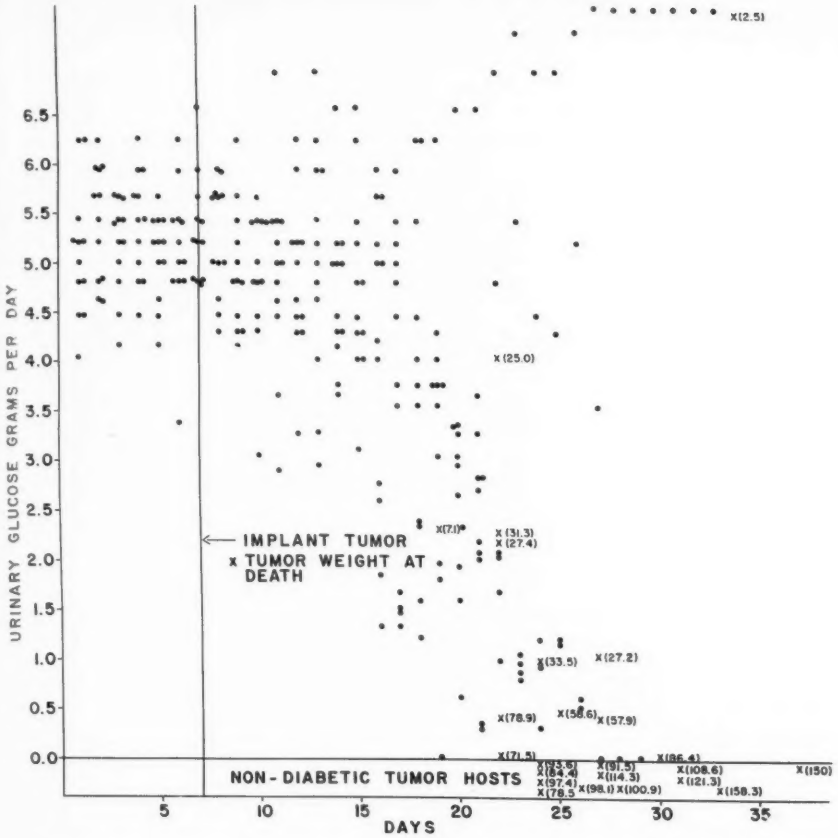

FIG. 3. Relationship between urinary glucose and tumor weight at time of death in force-fed rats. Individual values.

necrotic. The average time of survival of the non-diabetic tumor hosts was somewhat longer than for the diabetic tumor hosts.

Experiment 4 involved 12 normal rats and 24 extensively depancreatized rats. All of the rats were tube-fed the fluid diet. Each of the selected depancreatized rats excreted 6 to 7 gms. of glucose daily during the control periods. Twelve of the diabetic rats were treated with 15 units of regular insulin just after each morning and afternoon feeding. This treatment controlled the glycosuria. All of the rats were implanted with tumor and were killed at the end of 12 days. The average weights of tumors were as follows: normal rats, 38.8 ± 2.54 gms.: untreated diabetic rats, 15.1 ± 2.55 gms.; and insulin treated diabetic rats, 42.5 ± 2.80 gms.

Experiment 5 was a qualitative study of the possibility that the Walker carcinoma will completely degenerate in the severely diabetic rat prior to

the death of the host. Twenty-four rats which each had an average initial glycosuria of 6.5 to 8.0 gms. per day were implanted with tumor. After 14 days the tumors appeared very soft, and gross examination of the tumors of similar animals showed no recognizable viable tissue. The rats were then treated with 0.5 to 1.0 units of insulin per rat per day in an effort to sustain life in the host without rescuing the tumor. In each animal the tumor was reactivated and continued to grow until the animal died. A few such animals survived as tumor hosts for slightly longer periods than did non-diabetic rats, but the results were disappointing.

DISCUSSION

The diabetic tumor host represents two wasteful pathologic processes competing for calories and for protein; there is amelioration of the diabetes and retardation of the rate of tumor growth. Do the results require further explanation? There are other considerations which seem pertinent. The presence of insulin is required for at least some anabolic processes (6). Insulin is considered to be permissive for the anabolic effect of pituitary growth hormone. In other situations insulin behaves as though it must have a primary anabolic action (7). The severely diabetic rat is protein depleted. If it were possible to withdraw insulin abruptly from the treated protein-depleted depancreatized rat, it might then be possible to test the possibility that the tumor would grow normally in the absence of insulin if there were adequate amounts of protein to feed it. When we have attempted to do such experiments the force-fed animals have died in diabetic coma. Only gradual insulin withdrawal is tolerated under these conditions, and protein depletion occurs before insulin therapy can be completely stopped.

Goranson and Tilser (1) have suggested that the block in the entry of glucose into the metabolic cycle due to insulin lack may possibly restrict tumor growth by interfering with the supply of energy for the neoplastic process.

It seems probable that one mechanism by which the growing tumor can ameliorate diabetes is the utilization of exogenous and endogenous protein which would otherwise be used for gluconeogenesis. It is possible that the tumor also affects the utilization of carbohydrate within the tumor itself or in the peripheral tissues. Some other stressful conditions are known to ameliorate diabetes but the mechanisms are not known (8).

Jacobson (9) has reviewed the literature on the association of cancer and diabetes in man and has concluded that cancer occurs more frequently in diabetics than in non-diabetic patients. It is possible that the apparent greater-than-chance association of the two diseases represents selective factors which so frequently create spurious associations of relevant characteristics in clinical populations. It would be unsafe to infer that any causal connection between cancer and diabetes has been established.

Acknowledgment

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OVARIAN STIMULATION IN THE IMMATURE FOWL BY DESICCATED AVIAN PITUITARIES[1]

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ABSTRACT

Immature female brown Leghorn chickens were injected daily with a saline suspension of ground, acetone-dried anterior lobes of chickens. Significant follicular stimulation, but no precocious ovulation, was observed in those treated from 1 to 90 days of age, from 30 to 65 days, and from 60 to 150 days, but not in those treated from 1 to 35 days. All stimulated ovaries were essentially normal in appearance, showing none of the hyperplastic, cystic growth observed after treatment with pregnant mare serum (PMS) or mammalian follicle stimulating hormone.

Accelerated comb growth, indicating increased androgen secretion, was maintained in each group throughout the injection period. Such sustained comb stimulation cannot be produced by PMS, for cessation of growth and actual regression occur with prolonged treatment.

These results (follicular stimulation, general normality of the treated ovaries, sustained comb growth) support the hypothesis that there are qualitative differences between avian and mammalian gonadotrophins.

ALTHOUGH precocious follicular growth can easily be induced in many mammalian ovaries by treatment with gonadotrophic hormones, until recently (1, 2) little or no follicular stimulation had been observed in the immature avian ovary (See 3 for review of literature). In attempting to stimulate precocious follicular development in the chick, gonadotrophins from mammalian sources have been used almost exclusively. Taber (3) postulated that the lack of response might be due to inadequacies of the exogenous hormonal stimuli or to inherent unresponsiveness of the juvenile avian ovary.

Precocious endocrine function of the chick ovary can be produced by mammalian gonadotrophins, such as pregnant mare serum (PMS), but cannot be maintained. With continued treatment, complete refractoriness of the ovary, indicated by comb regression, occurs.

In connection with an entirely different problem (4) it was desirable to administer gonadotrophic hormones to ovariectomized chickens over a period of several months. It seemed possible that the use of avian gonado-

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trophins might eliminate some of the difficulties resulting from the use of mammalian preparations.

Several extracts of chicken pituitaries, including a crude suspension of acetone-dried anterior lobes, were tested for gonadotrophic activity in 21-day-old chicks. Since the gonadotrophic potency (as indicated by comb growth) was as great in the crude preparation as in the relatively purified extracts, extraction procedures were discontinued. After 15 days of daily treatments, combs (height×length) were 38% larger and ovaries 93% heavier than those of controls The ovaries of two of the five injected birds contained follicles over 0.5 mm. in diameter, whereas no follicles larger than 0.5 mm. were found in controls. Although the difference between follicular size in controls and pituitary-treated birds was not statistically significant, the results suggested that a more detailed study of the effects of chicken gonadotrophins on the immature chicken ovary should be made.

MATERIALS AND METHODS

The single-comb, light brown Leghorn was used in all experiments. Chicken heads, packed in ice, were obtained from a commercial poultry company[2] approximately 12 hours after decapitation. No attempt was made to obtain heads of the same variety, sex or age. The anterior lobes were removed, dried in acetone, frozen and stored until needed, at which time they were ground to a powder and the equivalent of 4 pituitaries was suspended in 0.5 cc. of saline. All injections were given subcutaneously, daily except Sunday. Controls and injected birds were kept in the same cages and body weights and comb sizes were checked once or twice a week. At autopsy, pituitaries, adrenals, thyroids and ovaries were weighed, fixed in formol-Zenker and prepared in the routine manner for hematoxylin and eosin-stained serial sections. In studying the ovaries, one section on every fifth row on the slides was carefully examined and these sizes of the three largest follicles were measured with an ocular micrometer. In addition, all follicles over 0.5 mm. in diameter were measured and counted on five sections, evenly spaced through the ovary. An indication of follicular size throughout each ovary as well as the diameter of the largest follicle could be determined by this method.

EXPERIMENTS AND RESULTS

General observations

Four groups of birds were treated: A, injected with 4 or 8 pituitaries from the day of hatching to 35 days of age; B, injected with 4 pituitaries, increased gradually to 16, from the day of hatching to 90 days; C, injected with 2–4 or 8–12 pituitaries from day 30 to day 65; D, injected with 12–20 pituitaries from day 60 to day 150.

In a few cases a mild, transitory, inflammatory reaction occurred at the site of the injection. Although the injected birds appeared healthy, their average body weights were less than controls, and significantly less in

[2] We gratefully acknowledge the cooperation and generosity of White Oak Acres Poultry Co., Monroe, North Carolina in supplying, packing, and delivering the chicken heads.

group A and in those receiving 2–4 pituitaries in group C (P = .01, .02, .04).

Accelerated comb growth, indicating stimulation of androgen secretion, was observed in all treated birds and, with the exception of three birds (one in C and two in D), this accelerated growth continued throughout the treatment periods.

In no group were there significant differences in adrenal or thyroid weights when compared with controls. However, increased vacuolization of thyroid follicles and decreased lipid content of adrenal cortical cells suggested increased thyroid and adrenal function and indicated the probable presence of thyrotrophic (TSH) and adrenocorticotrophic hormones (ACTH) in the pituitary powder.

Pituitary weights tended to be less in injected birds, and were significantly less in groups A and B (P = .05, .00, .01). No consistent histological differences could be detected in hematoxylin and eosin-stained sections.

In each treated group ovarian weight was greater than that of controls (Table 1). This increase was statistically significant except for the birds receiving 8 pituitaries in group A and the birds in group D. Although there were definite histological differences between the ovaries of treated and control birds, the treated ovaries were essentially normal in appearance. (Ovaries of group D were not sectioned.) In each group there was an increase in the number of polyovular follicles and an increase in the number of interstitial cells. Treated ovaries could easily be distinguished from controls on the basis of a relatively low proportion of vacuolated interstitial cells as compared with those with granular cytoplasm. There was a slight but definite increase in the thickness and density of the cortical tissue in treated ovaries, but little or no change in the medullary region.

A. *Chicks treated from 1 to 35 days of age*

Five chicks received 4 pituitaries (5.6 mg.) daily and 5 received 8 pituitaries (11.2 mg.) daily. Comb stimulation and increase in ovarian weight over the control weights (27% and 28%) were similar in the two injected groups. There was no increase in the size or number of follicles (Table 1).

An epidemic of Newcastle's disease occurred in the cage of birds receiving 8 pituitaries per day. Therefore, no valid comparison can be made as to the effectiveness of doubling the pituitary dosage. In spite of a severe weight reduction in the controls of this group, ovarian development and function were comparable to those of the healthy controls.

B. *Chicks treated 1 to 90 days*

Since no follicular stimulation was produced after 35 days of treatment, 5 chicks were injected for 90 days. The number of pituitaries given daily was gradually increased from 4 to 16 (5.6–25.2 mg.). Comb growth was

TABLE 1. OVARIAN RESPONSE TO CHICKEN PITUITARY POWDER

Treatment	No.	Comb (h×l)	Ovary (mg. S.D.)	% body wt.	Largest foll. (mm. S.D.)	No. foll. per sect.[1] .5+mm.	1.5+mm.	2.5+mm.	Polyovular foll. per sect.[2]
A. *Injected 1–35 days*									
4 pit.	5	6.0	125.5± 19.6	.05	.3± .07	0	0	0	1.6
Controls	4	2.0	98.5± 21.6	.03	.3± .06	0	0	0	.5
8 pit.[3]	5	6.0	96.2± 30.2	.05	.3± .04	0	0	0	5.2
Controls[3]	9	2.3	74.9± 16.2	.03	.3± .03	0	0	0	.7
B. *Injected 1–90 days*									
4–16 pit.	5	30.7	721.6±212.6	.09	2.7± .78	8.8	0.6	0.1	3.1
Controls	5	5.9	311.9± 3.6	.04	.7± .08	1.1	0	0	.6
C. *Injected 30–65 days*									
2–4 pit.	5	13.3	279.0± 63.6	.05	1.0± .57	1.8	0.3	0	1.2
8–12 pit.	7	18.0	370.6±124.2	.06	1.2± .59	3.9	0.1	0	.8
Controls	11	5.4	159.6± 31.8	.02	.6± .20	0.4	0	0	.3

[1] Averages from 5 evenly spaced sections.
[2] Averages from approximately every 30th section.
[3] Newcastle's disease in this group.

maintained at an accelerated rate throughout the period. Ovaries of injected birds were 132% heavier than controls and looked like normal ovaries of untreated pullets of about 5 months of age, just prior to the time of accelerated growth before the onset of laying.

The striking difference between control and treated ovaries was the difference in follicular size. All treated ovaries contained follicles over 1 mm. in diameter, more than twice the diameter of the largest follicle in any control; the average diameter of the largest follicle in the 5 treated ovaries was approximately 4 times that of the 5 controls. Follicular stimulation was observed throughout the ovaries and was not limited to one or two large follicles (see Table 1).

C. *Birds treated 38 to 65 days*

Since pronounced follicular stimulation was observed at 90 days, but none at 35 days, the following experiment was planned to determine if follicular stimulation could be produced prior to 90 days. Five chicks (Group 1) were given injections of pituitaries gradually increasing from 2 to 4 (2.8–5.6 mg.), and 7 chicks (Group 2) were similarly treated with pituitaries increasing from 8 to 12 (11.2–16.8 mg.), beginning day 30 and continuing to day 65.

Grossly, as well as histologically, the treated ovaries were normal in appearance. The major difference was in the size of follicles. In Groups 1 and 2, 80% and 86%, respectively, of the ovaries contained follicles larger than 0.5 mm. in diameter, as compared with 36% of controls; in Groups 1 and 2, 40% and 71%, respectively, contained follicles at least 1 mm. in diameter, as compared with 9% of controls (Table 1). In one treated ovary, there was a large number of lipoid-filled interstitial cells. This was the ovary from a bird which had shown comb regression after an initial stimulation.

D. *Birds injected 60 to 150 days*

This experiment was planned to determine if mature follicles and ovulation could be induced precociously. Six 2-month-old pullets were injected daily for three months with 12 to 20 pituitaries (16.8–28 mg.).

The response of the comb was not consistent in the injected birds. Two of the birds became refractory after two weeks of treatment. Both birds were smaller than the others and one was weak and sickly. In general, comb growth was accelerated, as compared with controls, for about two months. Early in the third month, with approaching sexual maturity, there was a significant increase in the rate of comb growth in controls, exceeding the relatively constant rate in injected birds. By the end of the experiment both comb size and the rate of growth were about the same in each group.

After 44 days of treatment, laparotomies were performed on three treated birds and two controls. Follicles measuring 1–3 mm. in diameter were observed in the treated birds, whereas no measurable follicles were seen in control ovaries.

Prior to autopsy on day 150, two controls and two treated birds laid, on days 141 and 150 and on days 145 and 148, respectively. There was no significant difference between weights of ovaries in the two groups, however the number of medium sized follicles in the ovaries of injected birds was considerably greater than in controls. In injected birds, 165[3] follicles from 2–17 mm. in diameter were counted, as compared with 63 in an equal number of controls. The number of follicles larger than 17 mm. was about the same: 8[3] in treated ovaries and 10 in controls.

DISCUSSION

Das and Nalbandov (1) were the first to report follicular stimulation in immature hypophysectomized and normal chickens treated with chicken pituitary powder. They observed such stimulation at 65 days of age, the youngest of their treated groups. Mammalian gonadotrophins failed to induce follicular growth. They concluded that the gonadotrophins from chicken pituitaries differed qualitatively from those of mammalian pituitaries. In an earlier paper on the response of males to gonadotrophins, Nalbandov, Meyer and McShan (5) postulated a third gonadotrophin, other than luteinizing (LH) or follicle stimulating (FSH) hormones, in avian pituitaries.

We observed three major differences between the effects of chicken pituitary powder and mammalian gonadotrophins. First, precocious follicular growth, similar to that reported by Das and Nalbandov, could be

[3] The actual figures for treated ovaries would be higher, for the largest ovary from the injected group was inadvertently destroyed before a complete count of follicles was made

stimulated by treatment with chicken pituitaries but not by treatment with PMS or mammalian FSH or LH (3). Failure to obtain follicular stimulation in the youngest group, 35 days old, suggests that the ovary at that age is not capable of responding.

Secondly, ovaries from birds treated with chicken pituitaries, although larger and more mature than those of controls, were essentially normal in gross and histological appearance. The normality of these ovaries was in striking contrast to the response observed after treatment with mammalian gonadotrophins. Typically, PMS and mammalian FSH produce pronounced medullary hyperplasia with distended medullary tubules. The result is a large, extremely cystic, abnormal organ. In the youngest group treated with chicken pituitaries the increase in ovarian weight over the controls was 27% as compared with a 400% increase in birds treated with PMS for the same period of time and having similar comb sizes. The increased number of polyovular follicles in the ovaries of birds treated with chicken pituitaries was similar to that observed following mammalian LH-treatment (3).

Thirdly, accelerated comb growth could be maintained in birds treated with chicken pituitaries, indicating that the ovaries did not become refractory to the treatment. In the chicks treated from the day of hatching (groups A and B) comb growth for 26 days was comparable to that of chicks receiving 100–140 I.U. of PMS. After this time, however, the combs of the pituitary-treated birds continued to grow at an accelerated rate, whereas comb regression occurred in the PMS-treated birds. In one or two-months-old birds treated with PMS refractoriness occurred after 12 days and in three-months-old birds cessation of comb growth and regression occurred after 8 days (3).

Our results support the hypothesis that the gonadotrophins of chicken pituitaries differ qualitatively from the mammalian. However, in both our experiments and in those of Das and Nalbandov, the entire anterior lobe of the chicken pituitary was used. In addition to gonadotrophins, this powder, in our experiments, was shown to contain some TSH and ACTH. Since the mechanism of follicular maturation, involving the mobilization of large amounts of yolk in the bird, is extremely complex, it is possible that other pituitary homones, in addition to gonadotrophins, play a role.

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NOTES AND COMMENTS

AUTOTRANSPLANTATION OF THE ADRENAL OF THE RAT TO THE PORTAL CIRCULATION: STUDIES IN MALE RATS[1]

ABSTRACT

Male rats were adrenalectomized and half of them castrated. Both major groups were subdivided as follows: One adrenal left *in situ*, one adrenal re-implanted in the systemic circulation, one adrenal re-implanted in the portal circulation with and without parenteral cortisone. Hypertrophy of the adrenal did not occur in any of the groups. This is in contrast to the findings in female rats, in which the intraportal adrenal underwent marked hypertrophy which was preventable by oophorectomy.

In a recent publication (1) it was demonstrated that an adrenal gland implanted in the portal venous bed of the female rat underwent an unusual hypertrophy that was preventable by oophorectomy. The present communication will demonstrate that this phenomenon does not occur in either the intact or castrated male.

METHODS

Male rats of the Long-Evans strain weighing approximately 250 gm. were divided into four groups of twelve animals each. In "Group A," which served as controls, the right adrenal gland only was extirpated. In the remaining three groups, after bilateral adrenalectomy, the left adrenal was re-implanted in a new site as follows: In "Group B" to the systemic circulation (i.e., the loose tissue around the spermatic cord); in "Group C" to the portal circulation (i.e., the greater curvature of the stomach adjacent to the splenic veins, rather than directly to the spleen where adhesions were more likely to occur); "Group D" was treated the same as "Group C" with the additional parenteral administration of cortisone, 1 mg. daily for 5 days of the week.

A second similar group of male rats were prepared to correspond to Groups A, B, C, and D described above, but in addition were castrated at the time of operation.

Following surgery, all of the animals were maintained on normal saline for a period of three weeks in addition to the standard laboratory diet. The rats were weighed, sacrificed and subjected to autopsy after 150 days. At autopsy, the adrenal gland was carefully freed of adherent tissues, blotted, and weighed. If adrenal remnants were found due to incomplete removal of the gland from its original site, or if accessory adrenal tissue was discovered, the animals were not included in the series.

RESULTS

From Table 1, it is evident that after unilateral adrenalectomy in the male rat, there is no significant compensatory hypertrophy of the remaining adrenal gland left *in situ*. Moreover, bilateral adrenalectomy and the re-implantation of one adrenal either to the systematic circulation or the the portal circulation, did not result in adrenal hypertrophy. This is in sharp contrast to what we previously demonstrated in the intact female rat (1), where it was found that the adrenal transplanted to the portal circulation showed a mean increment of 19.5 mg. per 100 gm. body weight which was more than twice that of the controls.

The data in Table 2 are not statistically different from those in Table 1, which im-

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TABLE 1. MEAN CHANGE IN ADRENAL WEIGHT AFTER 150 DAYS IN MALE RATS*

Type of experiment†	No. of rats	Initial wt.	Final wt.	Increment	S.D. of increment
		(Right adrenal)	(Left adrenal)		
A. Left adrenal *in situ*	11	5.1	6.5	1.4	1.7
B. Left adrenal in systemic circulation	10	6.3	7.4	1.1	3.5
C. Left adrenal in portal circulation	12	6.7	9.7	3.0	4.7
D. Left adrenal in portal circulation, parenteral cortisone 5 mg./week	11	6.4	10.0	3.6	3.1

* The weight of the adrenal is expressed as the mean weight in mg. per 100 gm. body weight.

† Group A was the only group in which unilateral adrenalectomy was performed. Groups B, C, and D were bilaterally adrenalectomized and the left adrenal re-implanted in a new site.

TABLE 2. MEAN CHANGE IN ADRENAL WEIGHT AFTER 150 DAYS IN CASTRATED MALE RATS*

Type of experiment†	No. of rats	Initial wt.	Final wt.	Increment	S.D. of increment
		(Right) adrenal)	(Left adrenal)		
A. Left adrenal *in situ*	10	5.8	7.8	2.0	1.4
B. Left adrenal in systemic circulation	12	6.5	8.3	1.8	4.8
C. Left adrenal in portal circulation	12	6.4	8.8	2.4	2.7
D. Left adrenal in portal circulation, parenteral cortisone 5 mg./week	10	7.0	10.1	3.1	4.2

* The weight of the adrenal is expressed as the mean weight in mg. per 100 gm. body weight.

† Group A was the only group in which unilateral adrenalectomy was performed. Groups B, C, and D were bilaterally adrenalectomized and the left adrenal re-implanted in a new site.

plies that castration had no demonstrable effect on the adrenal weights of male rats in any of these experiments. Similarly, the effect of cortisone is negligible.

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GONADAL INFLUENCES ON PLASMA FIBRIN AND FIBRINOLYTIC ACTIVITY: A POSSIBLE BASIS FOR THE FURTHER ANALYSIS OF SOME FORMS OF CORONARY THROMBOSIS

ABSTRACT

Analysis of plasma fibrin and fibrinolytic capacity in normal and gonadectomized male and female rats revealed male plasma fibrin to be statistically higher in males than in females, this difference being eliminated by gonadectomy due to elevation of fibrin after ovariectomy and the absence of change after orchidectomy. Fibrinolytic activity of plasma is the same in males and females under basal conditions, but is depressed by orchidectomy; it is especially low three hours after a fatty meal—but only in normal males.

On the basis of these findings an hypothesis is outlined to account for the higher incidence of coronary occlusion in males according to which the already susceptible males (with higher plasma fibrin) become even more susceptible to fibrin deposits when their plasma fibrinolytic capacity is markedly depressed after fatty meals with consequent diminished capacity to lyse fibrin deposits which may form in previously rigid and stenosed coronary arteries.

Fibrin deposition and plasma fibrinolytic activity have repeatedly been considered as important factors in the promotion or prevention of coronary thrombosis (2, 7). Considerable information is already available about blood lipid in relation to sex, diet, blood coagulation and heparin (8, 16, 17), and even scattered data relating to plasma fibrin and fibrinolytic activity as affected by sex (2) and by diet (14, 15). Yet, to our knowledge, there have not been any systematic studies of the relations between gonadal activity, plasma fibrin and fibrinolytic activity or of modifications thereof by fat intake (in relation to sex) in the production or prevention of coronary disease.

Our interest in this aspect of vascular physiology was initiated by our observation that if male and female rats, both groups with equivalent degrees of previously induced coronary sclerosis and stenosis (9, 10, 11), are placed on a high fat diet, then the males soon died while the females survived. However, males with comparable coronary lesions maintained on the stock diet, relatively low in fat, also lived until the time of sacrifice. As a working *hypothesis* we suggested that the males had died from acute coronary insufficiency occasioned by deposition, within already narrowed and rigid coronary arteries, of fibrin, the latter being precipitated by a sudden diminution of plasma fibrinolytic capacity following the consumption of a fatty meal in a manner described by Greig (14) and recently confirmed by us in rats (13). If this hypothesis can be proved then the thrombotic episodes in coronary disease will be better understood, especially in relation to sex differences and fat intake.

As the first step towards testing this hypothesis the plasma fibrinolytic capacity was examined in groups of normal and gonadectomized adult male and female rats under basal conditions. Subsequently the responses of plasma fibrinolytic activity and blood lipids, to single standard fatty meals, were also examined and reported fully elsewhere (13) as were the effects on these aspects of blood chemistry, of implanting estrogen and testosterone pellets into gonadectomized rats. Our present objective is primarily to report briefly the basal plasma fibrin and fibrinolytic activity in the above-mentioned groups of rats.

Fibrinolytic activity was estimated in duplicate for each rat, using modifications of the method of Biggs and Macfarlane (4) and Bidwell (3) permitting estimations to be conducted on tail blood. In essence this method involved the precipitation, by 10 units

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of thrombin (Parke, Davis)[1] of the fibrin present in 0.1 ml. of citrated plasma. Fibrinolytic activity was expressed as a percentage of the initial amount of fibrin, estimated in duplicate by the same method, on other 0.1 ml. samples of the same plasma after 4 hours incubation at 37° C.

While conducting these estimations, it was noted that the rate and quantity of fibrin formation (induced in citrated plasma by known quantities of thrombin) varied considerably in the different groups of animals. These differences were sufficiently gross to be obvious even macroscopically (Fig. 1). We therefore also estimated (in duplicate) the fibrin content of the plasma in these same group of rats—fibrin being expressed as tyrosine in 0.1 ml. of citrated plasma.

The results of our estimations of both plasma fibrin and fibrinolytic capacity in the various groups of animals under basal conditions are presented in Table 1 and Figures

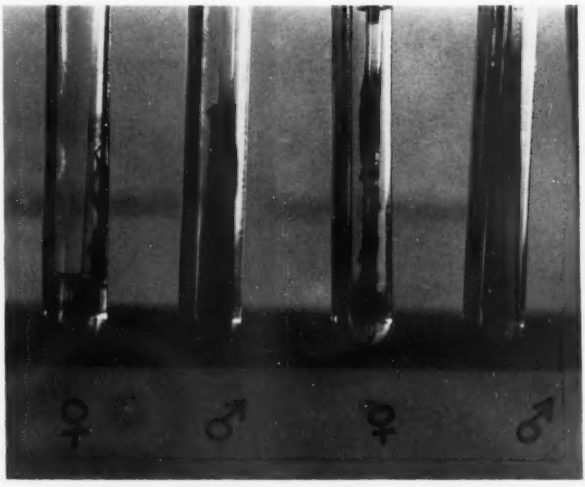

FIG. 1. *In vitro* fibrin yield from male and female rat plasma induced by Calcium Chloride. Note, much greater fibrin clot in "male" plasma. Each tube contained 0.4 mls. citrated plasma (male or female as indicated), 1.0 ml. of 0.9% Sodium Chloride, and 1.0 ml. of 1.1% Calcium Chioride.

2 and 3—the means and standard deviations being derived from analyses on 15 rats in each group.

The above data seem to permit the following conclusions: (1) The fibrin content of the plasma, as here determined, is significantly ($P < .01$) higher in normal male than in female Wistar strain albino rats. (2) These sex differences in plasma fibrin are virtually eliminated by gonadectomizing males and females, the elimination of the sex differences being due to increases in the fibrin content of the plasma of females 40 days after ovariectomy, while castration, of similar duration, produced virtually no change in male plasma fibrin levels (Table 1; Fig. 2). (3) The fibrinolytic activity of plasma, from normal male and female rats under *basal* conditions, do not differ statistically. (4) Castration of males is followed by profound, if somewhat variable, diminutions in plasma fibrinolytic

[1] We wish to thank Dr. Eugene H. Payne of Parke, Davis & Co., Detroit, for generous supplies of "Thrombin Topical" used in these studies.

TABLE 1. GONADAL INFLUENCES ON PLASMA FIBRIN YIELD AND FIBRINOLYTIC ACTIVITY IN RATS

		Normal	Castrated
Fibrin*	Males	49 ± 7	47 ± 14
	Females	28 ± 5	41 ± 6
Fibrinolytic activity†	Males	85 ± 9	59 ± 28
	Females	72 ± 15	77 ± 15

* Fibrin expressed as micrograms tyrosine/0.1 ml. citrated plasma.
† Fibrinolytic activity expressed as percentage lysis of initial clot after 4 hours incubation at 37° C.

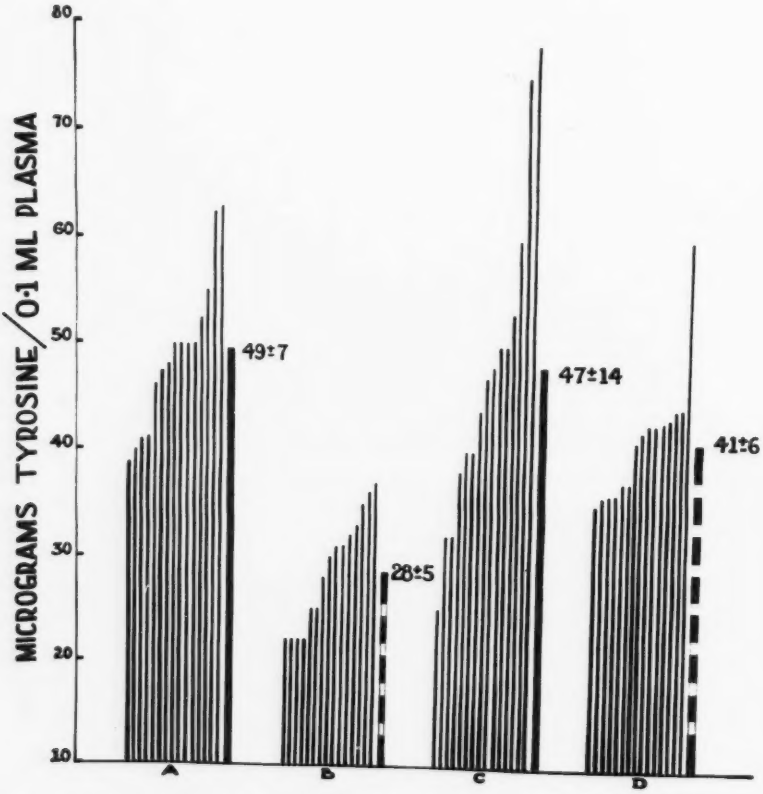

FIG. 2. Gonadal influence on fibrin yields from rat plasma (expressed as micrograms tyrosine 0.1 ml. plasma). A = Normal Males. B = Normal Females. C = Gonadectomized Males. D = Gonadectomized Females. Each line represents estimations in duplicate on one animal. Heavy lines represent means for each group of 15 rats (see Table 1 for actual values).

activity, the changes being statistically significant when compared with normal males ($P < .01$), whereas ovariectomy in females produces virtually no alteration (Fig. 3). Thus, the alteration in plasma fibrinolytic activity, induced by gonadectomizing male and female rats, is the complete inverse of the changes in the plasma fibrin content induced in the same rats by this operation.

Further, our preliminary studies of the clotting time, following recalcification, indicate that pooled plasma, from normal males or from ovariectomized females, clots at about the same speed (105–120 sec.) whereas that from normal females or castrated males both take between 200 and 215 seconds to clot. That the differences in amount and speed of fibrin yielded by plasma, in the different groups, using the above-mentioned techniques,

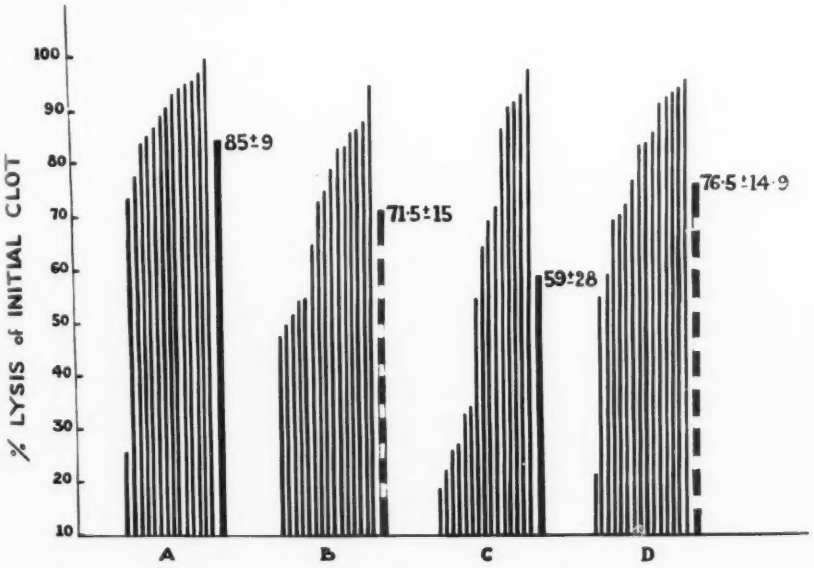

FIG. 3. Gonadal influence on fibrinolytic activity in rat plasma expressed as percentage of initial clot. A = Normal Males. B = Normal Females. C = Gonadectomized Males. D = Gonadectomized Females. Each line represents average of duplicate estimations on one animal. Heavy lines represent means for each group of 15 rats (see Table 1 for actual values).

were perhaps due to the presence of some powerful anticoagulant circulating in normal females also seems likely from our preliminary tests for such an anticoagulant using varying concentrations of thrombin. Studies of recalcification clotting times and of the existence of circulating anticoagulants are still being continued.

COMMENT

Since coronary pathology does not necessarily culminate in coronary occlusion (thrombosis) the need for understanding the mechanism for the *thrombotic* episodes themselves has been emphasized by Duguid (7) who considered that diet may perhaps not be important in this regard.

Our findings, recorded above, may constitute another step towards explaining the

preponderance of coronary failures in males as well as the apparent significance of fat intake in their production. In addition to the above data, however, we have now found, and reported on fully elsewhere (13), that male rats differ from females also in the degree of chylomicronaemia developed after a single standard fatty meal, despite the fact that, as shown by Deuel (6), sex differences in the *rate* of fat absorption are not demonstrable. These findings in rats conform with sex differences in chylomicronaemia, after standard fatty meals, previously described in man (1, 5). Moreover, the fibrinolytic activity of the plasma, three hours after a fat meal, has been found by us to drop to a far greater degree in male than in female rats, the degree of fall apparently being related to the intensity of chylomicronaemia which develops. As will be reported later, we have also found these differences in response to a fatty meal to be modifiable by gonadectomy and by hormone pellet implants.

It may well be that the lipoprotein particles in chylomicronaemic plasma, like other macromolecules, are phagocytized by the reticulo-endothelial system in a manner previously outlined (12). Such stimulation of the latter system might then be responsible for the observed fall in the plasma fibrinolytic capacity induced by fatty meals—especially in males.

If the tendency to deposit fibrin in vessels, prone to intimal "erosions" by pre-existing tortuousity and rigidity, is indeed determined in part by the fibrin content of plasma, on the one hand, and its fibrinolytic activity on the other, then, according to our presently reported findings, the order of such tendency would probably be first, castrated males, then normal males, next ovariectomized females and lastly normal females. Our additional data (13), indicate that this tendency will be intensified in males in whom chylomicronaemia and fall in plasma fibrinolytic activity after fat ingestion are both more marked than in females.

These findings certainly seem to provide a basis for studying further the interrelations between sex hormones, reticulo-endothelial activity, fat intake and apparent sex differences in fat absorption, transport and dissimilation in producing thrombotic episodes in patients with pre-existing coronary pathology.

Acknowledgments

We would like to express our gratitude to The Schlesinger Organisation and to the Wellcome Trust for generous financial assistance permitting the execution of this work.

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THYROID IODIDE CONCENTRATION IN ESTRADIOL-TREATED HYPOPHYSECTOMIZED RATS[1]

ABSTRACT

Treatment with α-estradiol benzoate produced no marked alteration of thyroid size or T/S ratio in hypophysectomized female rats, with or without concurrent thyrotrophin administration. The inhibitory effect of this estrogen on goitrogenesis does not appear, therefore, to be due to a direct action on the thyroid iodide concentrating mechanism.

In an attempt to localize the site at which estradiol acts to inhibit goitrogenesis (1), we looked for a direct influence of this hormone on thyroid iodide concentration. No such effect was seen in a pilot study[2] (unpublished) carried out in 1954. However, a subsequent report by Feldman (2) that treatment with another estrogen (estrone) increased the thyroid-serum radioiodide concentration (T/S) ratio of hypophysectomized rats four-fold, has led us to re-examine the question, repeating and extending our earlier experiment.

MATERIALS AND METHODS

Young hypophysectomized female rats, obtained from either the Hormone Assay Laboratories in Chicago, Illinois, or the Endocrine Laboratories in Madison, Wisconsin, were maintained from the third post-operative day on Purina Fox Chow, and 3% sucrose in tap water, *ad libitum*. They were weighed and divided into control and experimental groups on the 10th post-operative day. On this and the succeeding 5 days (except in Experiment VII, where a 3-day treatment period was used), the experimental rats were injected subcutaneously with 0.1 ml. of sesame oil containing the desired daily dose of the estrogen (α-estradiol benzoate).[3] In Experiments III–VI all animals were injected intraperitoneally with thyrotrophin,[4] dissolved in 0.5 ml. of saline, at either 12 hour (Exp. III) or 24 hour intervals throughout the 6-day treatment period. Drug dosages are shown in the table. On the 16th (in Experiment VII, the 13th) post-operative day, 12–16 hours after the last injection, T/S ratios were determined by the method of VanderLaan and Greer (3). Thyroid weights were measured to the nearest 0.1 mg. Body and adrenal weights were used to detect incomplete

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[1] This study was supported by grants B-708 and A-1396, National Institutes of Health.

[2] We are indebted to Dr. Richard A. Miller for hypophysectomizing the rats used in the preliminary study.

[3] We are indebted to Dr. Norman L. Heminway of Schering Corporation for supplying the α-estradiol benzoate.

[4] The thyrotrophin (lot $RxPO_4$ which assays at about 2 U.S.P. units/mg.) was generously donated by Dr. Wayne Donaldson of Parke, Davis and Company.

hypophysectomy, even if gross inspection of the operation site failed to reveal pituitary remnants.

RESULTS AND DISCUSSION

Mean relative thyroid weights, T/S ratios, and terminal body weights are presented in Table 1.

In the experiments in which no thyrotrophin was administered (I, II, and VII), the T/S ratios for estradiol-treated rats were slightly greater than those for their controls. However, the most striking increase noted (in group II, 50%) was not particularly significant ($P = .017$). There was no consistent effect on thyroid weight.

In Experiments III-VI, where the rats received thyrotrophin, the estrogen treatment failed to increase the T/S ratios. It even may have depressed them. On the other hand, relative thyroid weights appeared to increase, but this increase is probably due, in large

TABLE 1

Exp. No.	Estrogen, μg./day	TSH, mg./day	No. of rats	Body weight, gm.	Thyroid weight (mg./100 gm.)		T/S ratio	
					Control	Estrogen	Control	Estrogen
I*	—	—	9	145 ± 6.0	5.16 ± .25		6.1 ± .76	
	150	—	8	137 ± 4.2		5.19 ± .27	(p = .394)‡	6.9 ± .66
II†	—	—	5	140 ± 5.2	3.38 ± .32		5.8 ± 1.08	
	80	—	10	129 ± 2.7	(p = .064)	4.19 ± .10	(p = .017)	8.4 ± 1.01
III*	—	1.0	6	153 ± 3.2	5.92 ± .26		110 ± 7.4	
	150	1.0	6	139 ± 5.3	(p = .122)	6.80 ± .44	(p = .008)	76 ± 7.3
IV*	—	0.5	5	130 ± 3.3	4.96 ± .27		102 ± 9.1	
	150	0.5	4	124 ± 3.0	(p = .046)	6.03 ± .33	(p = .028)	71 ± 4.9
V†	—	0.25	6	145 ± 2.2	4.78 ± .24		85 ± 6.3	
	80	0.25	10	136 ± 1.7	(p = .655)	4.63 ± .20	(p = .161)	100 ± 7.7
VI†	—	0.0025	12	137 ± 2.0	4.32 ± .15		45 ± 5.2	
	80	0.0025	12	126 ± 1.9	(p = .221)	4.70 ± .26	(p = .138)	36 ± 2.7
VII†	—	—	12	139 ± 2.2	4.95 ± .34		3.5 ± .22	
	8	—	11	135 ± 1.1	(p = .329)	4.54 ± .29	(p = .337)	4.1 ± .63

* Rats obtained from Hormone Assay Laboratories, Chicago, Illinois.
† Rats obtained from Endocrine Laboratories, Madison, Wisconsin.
‡ Based on differences between control and estrogen values.

part, to the body weight loss invariably found in the estrogen-treated rats. These results do not suggest any significant modification, by estradiol, of the effects on thyroid size and iodide concentration of either .0025, .25, .5 or 1.0 mg./day of the thyrotrophin preparation used.

Thus, α-estradiol benzoate, in doses which inhibit goiter development in the intact rat (1), does not appear to greatly or consistently affect thyroid size or iodide "pumping" in the absence of the hypophysis. The action of this hormone in the intact goitrogen-treated rat is therefore not a direct one upon the iodide pump, although a direct effect upon some other phase of thyroid metabolism (i.e., incorporation of iodine into the thyroid hormone) is not ruled out by these experiments.

It is difficult to interpret the discrepancy between these findings and those of Feldman (2). In Experiment VII, the estrogen dosage (estradiol, 8 μg.) was comparable to that which he employed (estrone, 500 I.U.), but nothing approximating a four-fold increase in the T/S ratio was found. Therefore, it seems clear that any direct action estrone may have on the thyroid iodide "pump" should not be attributed to its estrogenic properties *per se*, since no such influence was exhibited by estradiol. A similar discrepancy between the effects of estradiol and estrone on thyroid I^{131} uptake has been noted in intact rats

(4). This suggests that these two estrogenic compounds may also differ in their ability to influence thyroid function in hypophysectomized animals.

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THE ENDOCRINE SOCIETY

1958 ANNUAL MEETING

The Fortieth Annual Meeting of The Endocrine Society will be held in the St. Francis Hotel, San Francisco, California, Thursday, Friday and Saturday, June 19, 20, 21, 1958.

The Committee on Local Arrangements is Dr. Roberto Escamilla, Chairman, Dr. Donald E. Bernstein, Dr. Peter H. Forsham, Dr. Minnie B. Goldberg, Dr. Gilbert S. Gordan, Dr. Francis S. Greenspan, Dr. C. H. Li as members of the committee.

All Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily in the Colonial Ballroom, and in addition there will be simultaneous afternoon sessions in the Italian Room. The annual dinner is scheduled for Friday, June 20th at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All reservations for rooms must be made through the AMA Housing Bureau, c/o Convention and Tourist Bureau, Civic Auditorium, San Francisco, California, after January 1, 1958. No reservations will be accepted before that date. If possible, official application forms as published in the Journal of the American Medical Association should be used. Arrival and departure dates must be stated. *This notice concerning procedure for making hotel reservations supersedes that published in the November 1957 issue of this journal.*

Final program, membership card and advance registration forms will be sent on May 15, 1958 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Rulon W. Rawson, 444 E. 68th St., New York 21, New York, not later than February 1, 1958. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program:

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.

2. The title heading must be arranged as follows:

Line 1. Title, not to exceed fifteen words;

Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.

Line 3. Institution of origin and city in which institution is located.

3. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.

4. Abstracts should be letter perfect, since there will be no opportunity for proofreading by the authors.

ERRATUM

In Tables 3 and 4 in the article "Ketogenic and Adipokinetic Activities of Pituitary Hormones" by Frank L. Engel, Mildred G. Engel and Harry T. McPherson which appeared in *Endocrinology* **61**: 713, 1957 (December) all the ditto marks should be removed from under "0.1*N* NaOH—24 hours."

AMERICAN CANCER SOCIETY

The American Cancer Society has announced that Clinical Fellowships at the senior resident level for the academic year 1959–60 may be applied for by institutions accredited by the Council on Medical Education and Hospitals of the American Medical Association to give training in the following specialties and sub-specialties, with emphasis on the diagnosis and treatment of cancer: internal medicine, malignant diseases, neurological surgery, obstetrics-gynecology, orthopedic surgery, otolaryngology, pathology, public health, radiology, surgery, and urology.

Institutions will be notified of awards granted in June 1958. Individual candidates should apply directly to an institution, or the American Cancer Society, for information concerning fellowships. The annual stipend, tax exempt, is $3,600.

Application forms are available from the Director of Professional Education, American Cancer Society, Inc., 521 West 57th Street, New York 19, New York.

February 15, 1958 is the deadline for institutions submitting applications for the 1959–60 clinical fellowships.